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Impact of disease in dairy cows on ceftiofur pharmacokinetics, withdrawal times and emergence of antimicrobial resistance

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Impact of disease in dairy cows on ceftiofur pharmacokinetics, withdrawal times and emergence of antimicrobial resistance

by

Patrick John Gorden

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Veterinary Microbiology

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The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this dissertation. The Graduate College will ensure this dissertation is globally accessible and will not permit alterations after a degree is conferred.

Iowa State University

Ames, Iowa

2017

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DEDICATION

I dedicate this dissertation to those who have given up so much for me to complete this degree. First of all, to my amazing wife Kelly who has been remarkably tolerant and supportive of the long hours with me away from home and our family in order to complete this work while being fully employed. Secondly, to my kids, Sam, Abby, and Erica. I realize there are things you would have rather done that hang around town during school breaks while Dad finished yet another project. I hope you realize that everything that I have done since you were born was to make a better life for you. Finally, to my parents, who instilled the work ethic in me by working so hard while I was growing up, to give me better opportunities than you had. I know that it was important for you two to give all of your children the opportunity to get a good education and become successful citizens of Iowa and the world. To all of you, thank you for all that you have done through the years to get me to this point. I couldn't have done it without your love and support!

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NOMENCLATURE

AMDUCA	Animal Drug Use Clarification Act
AMR	Antimicrobial Resistance
AUC	Area Under the Curve
CDC	Center for Disease Control
CE	Ceftiofur Equivalents
CEF	Ceftiofur
CFA	Crystalline Free Acid
cfu	colony forming unit
CL	Clearance
CL/F	Apparent systemic clearance
CM	Clinical Mastitis
C _{max}	Maximum plasma concentration
CVM	Center for Veterinary Medicine
d3-CEF	Deuterated Ceftiofur
DCA	Desfuroylceftiofur Acetamide
DFC	Desfuroylceftiofur
ESBL	Extended Spectrum β -Lactamase
F	Absolute bioavailability
FAO	Food and Agriculture Organization of the United Nations
FDA	Food and Drug Administration
FSIS	Food Safety and Inspection Service

IM	Intramuscular
IMM	Intramammary
IV	Intravenous
KIS	Kidney Inhibition Swab
LC-MS	Liquid Chromatography coupled with Mass Spectrometry
MDR	Multi-Drug Resistant
MIC	Minimum Inhibitory Concentration
MRM	Multiple Residue Method
MRT	Mean Residence Time
NAHMS	National Animal Health Monitoring Survey
NARMS	National Antimicrobial Resistance Monitoring System
NTS	Non-Type Specific
OIE	World Organization for Animal Health
OTC	Oxytetracycline
PCR	Polymerase Chain Reaction
PK	Pharmacokinetics
ppb	Parts per billion
ppm	Parts per million
PR/HAACP	Pathogen Reduction/Hazard Analysis and Critical Control Point
PRRSv	Porcine Reproductive and Respiratory Syndrome virus
SPE	Solid Phase Extraction
$T_{1/2}$	Half-life
$T_{1/2 \lambda_z}$	Terminal half-life

T_{\max}	Time of maximum concentration
UDSA	United States Department of Agriculture
V_D	Volume of Distribution
V_D/F	Apparent volume of distribution during the elimination phase
V_{ss}	Volume of distribution at Steady State
WHO	World Health Organization

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ABSTRACT

Producing safe and wholesome food is a priority of animal agriculture in order to maintain consumer confidence in the products produced by the animal agriculture industries. Antimicrobial residues and antimicrobial resistant organisms are two of the most important concerns of consumers regarding food safety. Withholding periods are in place to minimize the risk of antimicrobial residues being present in food. However, these withholding periods are established on healthy animals, not clinically ill animals. Additionally, there is no such withholding period in place to minimize the risk of transporting antimicrobial resistant organisms through food following treatment, although it has been proposed. The first objective of this dissertation was to investigate the impact of clinical disease on the pharmacokinetics of ceftiofur. The second objective was to investigate changes in the fecal microbiota following drug therapy. The results of this work demonstrate the volume of distribution is increased in diseased animals and area under the concentration curve is decreased. Additionally, the mean elimination half-life can be significantly different. In both studies involving clinical disease, at least one animal in each group had an elimination half-life that was nearly twice as long as the mean of the control group. No animals were found to have violative drug residues present in tissues following observation of the labeled withholding time. Fecal *Escherichia coli* populations transiently decreased following therapy with ceftiofur and ceftiofur resistant populations were significantly different than untreated controls. There was a tendency towards a significantly higher ceftiofur resistant *E. coli* population in diseased animals treated with ceftiofur versus healthy animals treated controls. By 14 days following therapy, total and resistant *E. coli* populations returned to pre-treatment levels. *E. coli* isolates

that were resistant to ceftiofur were also cross-resistant to ampicillin and ceftriaxone. Additionally, 64.3% of the resistant isolates were also resistant to tetracycline. The β -lactamase gene *bla*_{CTX-M} was most commonly found in ceftiofur resistant isolates but mechanism for ceftiofur resistance was not identified in 55.8% of the ceftiofur resistant isolates. Isolates that were determined to be phenotypically ceftiofur cross-resistant were resistant to 4.72 while isolates that were ceftiofur sensitive were cross-resistant to 1.1 antimicrobials. The current study suggests that changes in bacterial populations following clinical disease are not different from those of healthy cows treated with ceftiofur. Observation of the established drug withholding period following treatment with ceftiofur crystalline free acid minimizes the risk of transferring fecal isolates harboring antimicrobial resistance to the public.

CHAPTER 1. LITERATURE REVIEW

Cephalosporin Residues in Dairy Cattle

Pharmacology of ceftiofur

Ceftiofur (CEF) is a 3rd generation cephalosporin approved by the Food and Drug Administration (FDA) in the United States (US) for cattle, swine, horses, sheep, goats, dogs, day-old chickens, and day-old turkey poults, depending upon the formulation. In the US, there are currently 5 veterinary formulations of CEF that could be utilized in dairy cattle, all of which were developed and are now marketed by Zoetis, Inc (Florham Park, NJ) or their predecessor companies. These include three parenteral formulations, CEF sodium (Naxcel[®]), CEF hydrochloride (Excenel[®] RTU EZ), and CEF crystalline free acid (CFA) (Excede[®]). There are also two intramammary (IMM) preparations, both of which contain different concentrations of CEF hydrochloride (Spectramast[®] LC and Spectramast[®] DC). In addition, there is now a generic formulation of CEF sodium (CeftiFlex[®]; Cephalone Pharma LLC, Pomona, CA). In the US, CEF is the only cephalosporin approved for parenteral use in the bovine.

As it is a 3rd generation cephalosporin, CEF is considered a broad-spectrum antimicrobial. In the US cattle industry, parenteral CEF products are labeled for bovine respiratory diseases (shipping fever and pneumonia) associated with *Mannheimia haemolytica*, *Pasteurella multocida*, and *Histophilus somni*; bovine interdigital necrobacillosis (foot rot, pododermatitis) associated with *Fusobacterium necrophorum* and *Bacteroides melaninogenicus*; and acute metritis associated with organisms susceptible to CEF in cows 1-14 days postpartum. It should be noted that not all formulations are labeled for all of these listed indications. For example, CEF sodium is not labeled

for the treatment of acute metritis. Usage of CEF sodium for the treatment of acute metritis would be considered a violation of the Animal Medicinal Drug Use Clarification Act (AMDUCA), as there is already a labeled formulation of CEF approved for acute metritis in CEF hydrochloride and CEF CFA (US FDA, 1996).

The lactating IMM product (Spectramast[®] LC) is labeled for clinical mastitis caused by coagulase negative staphylococci, *Streptococcus dysgalactiae*, and *Escherichia coli*. In addition, it is labeled for diagnosed subclinical infections associated with coagulase negative staphylococci and *Streptococcus dysgalactiae*. Finally, the dry cow IMM formulation (Spectramast[®] DC) is labeled for subclinical infections caused by *Staphylococcus aureus*, *Streptococcus uberis*, and *Streptococcus dysgalactiae* (Zoetis Services LLC, 2017).

Cephalosporins exert their activity by binding to penicillin-binding proteins and disrupting the developing bacterial cell wall. Like other β -lactams, they are considered to be bactericidal, although not as bactericidal as carbapenems and penicillins. Cephalosporins are time above minimum inhibitory concentration (MIC) dependent killers, so maintaining an effective plasma concentration for a sufficient duration is more important than creating a high peak concentration. Being broad spectrum antimicrobials, cephalosporins generally have good activity against *Staphylococcus*, including β -lactamase producing strains; streptococci; gram-negative organisms, with the exception of *Pseudomonas*; and anaerobic bacteria, except *Bacteroides fragilis*. Cephalosporins also do not have activity against *Enterococcus* spp. Third generation cephalosporins generally have less activity against gram-positive organisms, although there is much variability in the group. Cefotaxime is considered to have the best activity against streptococci amongst the 3rd generation drugs (Papich and Riviere, 2009).

Following systemic absorption for the administration site, CEF is rapidly metabolized to its active metabolite, desfuroylceftiofur (DFC) and small concentrations of other non-polar and polar metabolites (Jaglan et al., 1989; S. A. Brown et al., 1991). Desfuroylceftiofur has an exposed sulfhydryl group that forms reversible disulfide bonds with other sulfurous compounds, such as sulfhydryl-containing proteins and glutathione. Cephalosporins are relatively polar compounds and generally have a low volume of distribution (V_D), usually 0.2-0.3 L/kg (Papich and Riviere, 2009). In cattle, protein binding is age-dependent, with calves often having <50% of the CEF metabolites being protein-bound while healthy adult cows have CEF metabolites that are >90% protein-bound (S. A. Brown et al., 1991). This increases the excretion half-life ($T_{1/2}$) compared to most other cephalosporins, which are generally considered to have a short $T_{1/2}$. Ceftiofur CFA is a slow release compound due its ability to form a depot at the site of injection, thus giving it prolonged periods of activity compared to CEF sodium or CEF hydrochloride (Papich and Riviere, 2009).

Repeated dosing with CEF may result in saturation of protein binding sites, resulting in the presence of more unbound, active metabolites over time without increasing $T_{1/2}$ (Halstead et al, 1992). More than 95% of administered CEF sodium is excreted within 24 hours; with 61-77% of the total drug excreted through urine and the balance excreted in feces (S. A. Brown et al., 1991).

Drug approval studies show that metabolism and excretion of CEF hydrochloride by cattle is similar to that of CEF sodium (US FDA, 1998). Tissue distribution of CEF is similar regardless of the CEF salt administered (US FDA, 1998; KuKanich et al., 2005). Desfuroylceftiofur has clinical efficacy similar to cefotaxime (Papich and Riviere, 2009). However, DFC does not have good clinical activity against *Staphylococcus* in comparison to parent CEF (Cortinhas, 2013).

Cephalosporin use in the dairy industry

Antimicrobial use in the US dairy industry, or the US livestock industry, is not well documented (Landers et al., 2012). This is a source of controversy regarding the impact on public health (Mellen et al., 2001; Pew Commission, 2008). According to the US FDA, 34.34 million pounds of antimicrobials were sold for veterinary use in 2015. This is a 24% increase over 2009 data. Of the total, 13.05 million pounds of ionophores and other antimicrobial that are not medically important were sold, representing 38% of the total sales (US FDA, 2016a). The remaining sales represented medically important antimicrobial drugs, as dictated by the US FDA (US FDA, 2003). Tetracyclines represented the largest share of the 21.29 million pounds of medically important antimicrobials sold at 71% of the total, while fluoroquinolones and cephalosporins each represented <1%. Feed grade antimicrobial sales accounted for 70% of the sales, water-soluble medications were 24%, injectable medications were 5%, and other routes were 1%. Intramammary antimicrobials are included in the “Other” category (US FDA, 2016a). Sales figures for 2016 are not yet available, but it is expected that sales will be increased again in 2016 with the implementation of the Veterinary Feed Directive on January 1, 2017 (US FDA, 2013), as many entities stockpiled feed grade antimicrobials prior to the implementation date.

Based on the 2015 sales, the dairy industry utilizes a share of the 1.06 million pounds of injectable medications and 0.21 million pounds from the other routes category. Since sales are tracked through drug distribution networks that supply all facets of the livestock industry, more precise data is not available. The US FDA recently proposed a biomass calculator to more precisely estimate drug use by each of the major livestock species (US FDA, 2017).

Every 5-7 years, the US Department of Agriculture (USDA) conducts a National Animal Health Monitoring Survey (NAHMS) of the dairy industry. The most recent dairy survey was

undertaken in 2014. According to one of the reports that specifically looked at milk quality, 24.8% of all lactating animals have been affected with mastitis during the 12 months prior to the survey, with 87.3% of all clinical cases treated with an IMM antimicrobial. In total, 89.4% of operations treated mastitis with antimicrobials. In addition, 48.4% of participating operations reported using systemic antimicrobials to treat mastitis. In all total, 96.9% of operations used an antimicrobial via any route to treat mastitis. β -lactam antimicrobials were utilized by 85.6% of operations, with cephalosporin antimicrobials representing the majority of the total β -lactam use. Intramammary tubes containing CEF were used as the primary IMM antimicrobial on 38.6% of operations, while IMM tubes containing cephalixin were utilized on 34.4% of all operations. When the survey was broken down by treatments to the cow level, 50.4% of cows were treated with CEF, while 15.2% were treated with cephalixin. This indicates that large herds (≥ 500 cows) in this study used CEF as their primary IMM antimicrobial. Regarding antimicrobial selection, only 22.2% of operations utilized bacterial culture prior to deciding which antimicrobial to utilize (USDA, 2016a).

Other disease maladies in adult dairy cattle investigated by the 2007 NAHMS dairy survey included respiratory disease, diarrhea/digestive problems, reproductive diseases, lameness, and other. Respiratory disease affected 2.9% of cows, with 96.4% of those cases treated with an antimicrobial. Thirty-three percent of respiratory cases were treated with cephalosporins. Diarrhea affected 6% of cows on US dairies, with 32.3% of those cases treated with an antimicrobial. The majority of these cases were not treated with an antimicrobial; however, when they were, 11.3% were treated with a cephalosporin. Reproductive disease affected 10% of the cows, with 74.7% treated with an antimicrobial. Cephalosporins were used 17.2% of the time for these cases. Lameness affected 12.5% of cows, with 56.5% treated with antimicrobials; while “other” diseases

affected 0.7% of cows, with 66.2% of those treated with antimicrobials. Cephalosporins were used for 23% and 1.8% of the lameness and other categories, respectively (USDA, 2008a).

In a more recent survey of Midwest dairy farm treatment practices, our research group investigated drug use on 85 dairy farms. As with the NAHMS study, mastitis represented the most common reason for antimicrobial use in lactating cows. In our study, 100% of farms were using antimicrobials to treat mastitis, with 85% of farms indicating that cephalosporins were the primary drug class utilized. Sixty percent of farms indicated that CEF was their primary IMM choice for mastitis therapy, followed by cephalixin (25%). This finding was similar to that reported in a survey of large Wisconsin dairy farms, in which 71.6% of cases were treated with IMM CEF as the primary treatment (Oliveira and Ruegg, 2014). Older studies have found that cephalixin was the most commonly used IMM antimicrobial (Sawant et al., 2005; Raymond et al., 2006); however, these studies were conducted prior to the approval of the IMM formulation of CEF (US FDA, 2005). Fifteen farms in this survey indicated that they used bacterial culture to dictate therapy. Only three farms chose to not treat some cases of mastitis based on culture results (Schuler et al., 2017).

In the previously mentioned survey of Midwest farms (Schuler et al., 2017), treatment practices in lactating dairy cows were recorded for systemic mastitis, metritis, respiratory disease, and lameness; in addition to IMM mastitis therapy. Ceftiofur was indicated as the primary antimicrobial choice on 22%, 88%, 74%, and 90% of the farms for systemic mastitis, metritis, respiratory diseases, and lameness therapy, respectively. Reasons for selection of CEF as the primary antimicrobial for these diseases were the short milk withdrawal; broad spectrum of activity; and maintaining on-label therapy for metritis, respiratory diseases, and foot rot (Schuler et al., 2017). These findings are consistent with previous studies (Zwald et al., 2004; Sawant et al.,

2005; Raymond et al., 2006), confirming that CEF is the most commonly used antimicrobial on dairy farms in the US.

Dry cow IMM therapy represents the most common preventative use of antimicrobials in adult dairy cattle, although there is also therapeutic benefit to dry cow therapy (Arruda et al., 2013a,b; Johnson et al., 2016). The 2014 NAHMS study determined that 90.8% of operations dry treat at least some of the cows on their operations and 80.3% of the farms treat all cattle that concluded a lactation with IMM antimicrobials. In total, 93% of all cows in the survey received dry cow therapy at the end of their lactation (USDA, 2016a). Cephapirin was the most commonly utilized dry cow therapy in the NAHMS survey, with 58.1% of operations utilizing this product, followed by CEF on 27.9% of operations. However, like with lactating IMM products, larger farms in this dataset utilized CEF as their dry cow therapy (USDA, 2016a). In the survey of Midwest dairy farms, CEF and cephalixin were each used on 41% of the farms surveyed (Schuler et al., 2017).

Based on this information, it is readily apparent that the US dairy industry has a heavy reliance on the use of CEF. Surveys that have investigated the reasons why dairymen select one antimicrobial over another indicate that past history is the most common determinant, with a veterinarian being consulted in <50% of cases (Sawant et al., 2005; USDA, 2016a). Additionally, short withdrawal intervals for meat and milk make CEF an attractive antimicrobial choice, as the cost of discarded milk is often the most expensive component of drug therapy in lactating dairy cattle. Finally, marketing strategies promoting broad spectrum therapy and flexible labeling to maintain on-label therapies for the IMM product, have made CEF an attractive choice for

veterinarians and dairy producers. However, with increasing levels of antimicrobial residues associated with CEF use and concerns about antimicrobial resistant bacteria being transmitted to humans, there are fears that application of drug stewardship and prudent drug use practices are not being followed (US FDA, 2012b). Ceftiofur is closely related to the human drug ceftriaxone and resistance mechanisms can be mediated by similar mechanisms between the two antimicrobials (see below).

Antimicrobial residues in milk and dairy beef from cull dairy cows

Milk and meat offered for sale by dairy producers are routinely screened for violative antimicrobial residues. For Grade “A” milk, which represents >99% of all the milk produced in the US, every tanker truck load of milk is screened for β -lactam antimicrobials using ELISA technology prior to being offloaded for processing. These test kits have sensitivity levels that detect drug residues at or below US residue tolerances. These tests are not quantitative nor do they identify the individual β -lactam antimicrobial that is present in the milk (US FDA, 2015). Using this approach, the US dairy industry has achieved historically low levels of tanker truck loads with violative β -lactam residues (Figure 1). The most recent data indicated that only 0.011% of tankers contained violative levels of β -lactams (GLH Inc., 2016). Beginning July 1, 2017, a residue testing program was implemented for screening tanker trucks for tetracycline family drugs (US FDA, 2015). Currently, there is no data available regarding violative tetracycline residues.

Violative meat residues are monitored by a combination of sampling approaches conducted by the USDA. Tier 1 sampling is random in nature and is used to determine the baseline level for chemical residue exposure to consumers from meat products. The number of samples collected in each production class is based on the probability of finding at least one positive sample. The

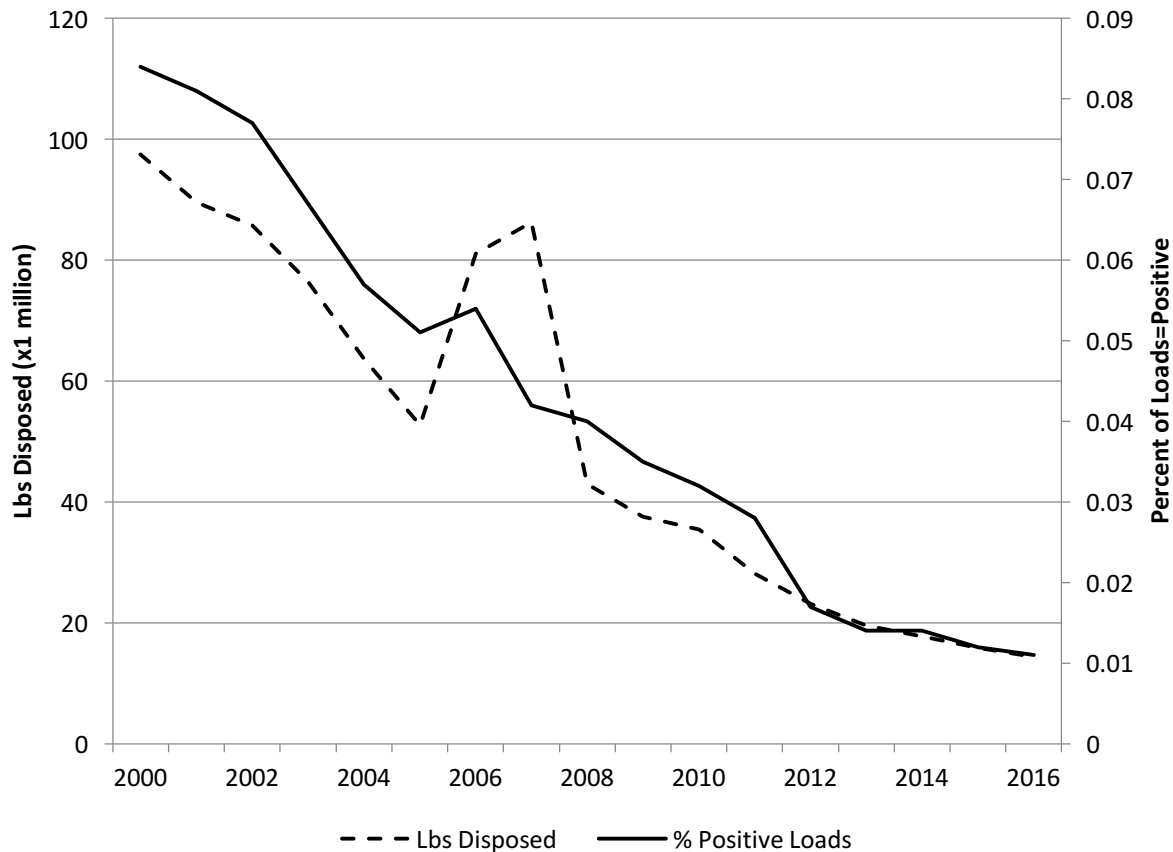


Figure 1. Percentage of tanker truck loads of Grade “A” found to have violative antimicrobial residues for β -lactam antimicrobials and total volume of milk disposed (2000-2016). Source: National Milk Drug Residue Data Base (GLH Inc., 2016).

analyses performed on the samples is dependent upon the production class and laboratory capacity.

The samples may be tested with one or more of the following methods: the Multiple-Residue Method (MRM), aminoglycoside method, pesticide method, metal method, β -agonist method, hormones method, avermectins method, or arsenic method. The MRM methodology can determine the presence and quantify residues of approximately 90 analytes simultaneously, not just antimicrobials, using liquid chromatography coupled with mass spectrometry (USDA, 2016b).

In FY2016, there were 739 Tier 1 samples collected in cull dairy cattle, with three violative samples found. Two of these violative samples were caused by sulfadimethoxine and one was caused by a permethrin insecticide. Compared to the beef cull cow production class, these numbers are comparable (USDA, 2017b).

The second sampling approach is Inspector-Generated Sampling. Inspector-generated samples are initiated by in-plant veterinary inspectors, when they detect evidence of disease that they suspect has been treated with a drug or just suspect a drug has been administered. In these circumstances, the Kidney Inhibition Swab (KISTM) test (Charm Sciences Inc., Lawrence, MA) is utilized in-plant. If the KISTM test is positive, samples from the suspect animal are submitted to a USDA laboratory, where the MRM methodology is utilized to determine the presence of and quantify residues (USDA, 2016b).

In addition to the testing described above, USDA implements targeted testing in response to data obtained in the above testing programs or from information received by the FDA or Environmental Protection Agency regarding misuse of animal drugs and/or exposure to environmental contaminants. The nature and degree of testing is dependent upon each situation (USDA, 2016b).

During the FY2016, USDA conducted 99,660 Inspector-Generated KISTM tests on cull dairy cattle and found 2276 positive results. In cattle, there were 129,522 total KISTM tests conducted in FY2016 (USDA, 2017b). In CY2016, there were 30.1144 million cattle processed in US, of which, there were 2.8857 million dairy cattle processed. Despite cull dairy cows only accounting for 9.6% of the total US marketed cattle (USDA, 2017c), they account for nearly 77% of the KISTM tests run in 2016 (USDA, 2017b). The rate of inspector-generated samples is triggered: 1) by the incidence of previous residue positive samples; and 2) by the presence of

carcass defects. Observations of animals that are marketed with mastitis, metritis, pneumonia, peritonitis, surgical incisions, or active injection site lesions may generate a screening test for antimicrobial residues (USDA, 2016b).

As a result of MRM testing from inspector-generated samples, there were 480 dairy cull cows with confirmed residue violations and 574 total residues detected in FY2016. In all cattle, there were 591 animal carcasses and 740 total violative residues detected. This means that during FY2016, cull dairy cattle accounted for 81.2% and 77.6% of the total cattle with confirmed residue violations and total residues confirmed, respectively (USDA, 2017b).

Desfuroylceftiofur, the official marker residue for CEF, was the most commonly found residue with 192 violative residues present in FY2016, followed by penicillin with 153 confirmed violative residues. There were also violative residues present for: sulfadimethoxine (67), flunixin meglumine (49), ampicillin (28), sulfamethazine (27), florfenicol (11), oxytetracycline (8), tilimicosin (8), lincomycin (5), gentamycin (4), sulfadoxine (4), meloxicam (3), dihydrostreptomycin (3), ketoprofen (2), neomycin (2), tylosin (2), and one violative residue each for amikacin, cefazolin, ciprofloxacin, sulfmethoxazole, sulfamethoxypyridazine, and tetracycline (USDA, 2017b). The presence of sulfamethazine, ciprofloxacin, sulfadoxine, sulfmethoxazole, and sulfamethoxypyridazine indicate that illegal drug use took place, as these are all prohibited drugs in dairy cattle. In addition, the presence of florfenicol, tilimicosin, lincomycin, gentamycin, meloxicam, ketoprofen, neomycin, tylosin, amikacin, and cefazolin would be violations of AMDUCA, as these would have to be used in an extra-label manner in dairy cattle. The stipulations within AMDUCA state that if a veterinarian is going to prescribe a drug in an extra-label manner, they must prescribe a withdrawal time that is sufficiently long to prevent a violative residue from being present in meat or milk when offered for sale for human consumption (US FDA, 1996).

Figure 2 displays the residue violation data for the last ten years in cull dairy cattle. In 2007, no violative residues for CEF were detected in cull dairy cattle. From 2008 to 2012, the number of violative CEF residues ranged from a low of 53 in 2011 to a high of 124 in 2009. Beginning in 2012, there was an increasing trend of CEF violative residues in cull dairy cows, with 130 in 2012, 238 in 2013, and 283 in 2014, which was the highest number recorded over the last 10 years. It should be noted that in 2013, the FDA changed the time frame for reporting residue data from a calendar year to a fiscal year. As a result, 2013 data only represents 9 months of time (USDA, 2015a). In 2013, CEF surpassed penicillin as the most common residue violation found in cull dairy cattle and has remained the highest violative residue analyte through FY2016 (USDA, 2008b, 2009, 2011b, 2012a, 2013, 2014, 2015a, 2015b, 2017a, 2017b).

Potential reasons for the increase in CEF violative residues include a short slaughter withdrawal, changes in tolerances for CEF in processed tissues, and changes in testing methodology implemented by the USDA. When used according to label, the parenteral products have a zero-hour milk withdrawal. Ceftiofur sodium and CEF hydrochloride formulations currently have a 4-day slaughter withdrawal, while CEF CFA has a 13-day slaughter withdrawal. When CEF sodium was first approved in 1988, it had a zero-day slaughter withdrawal, as there was no need to establish a tolerance as the residue levels in tissues were below the safe concentration with a zero-day withdrawal because the drug is rapidly cleared from the body (US FDA, 1988). However, with the approval of the first CEF hydrochloride parenteral formulation in 1998, tissue residues did not clear as readily as with CEF sodium and subsequently a tolerance of 8 parts per million (ppm) was established by the FDA. Desfuroylceftiofur, was designated as the marker residue with kidney being the marker tissue (US FDA, 1998). This change resulted in implementation of a 2-day slaughter withdrawal for CEF sodium and parenteral CEF

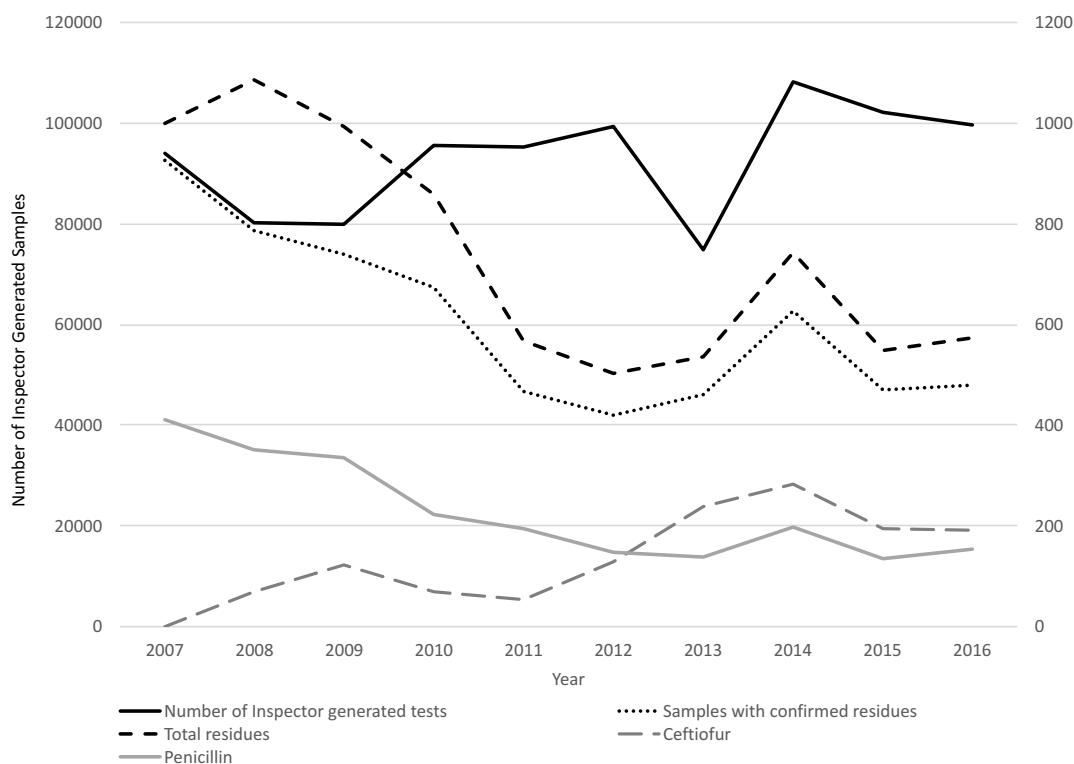


Figure 2. Summary of residue sampling and results for cull dairy cattle (2007-2016). The number of Inspector-Generated Kidney Inhibition Swab (KISTM) tests conducted each year is displayed on the left axis. Total violative residue detected, number of carcasses with violative residues, number of violative ceftiofur residues, and number of violative penicillin residues are displayed on the right axis. The years 2007-2012 were based on calendar years. In 2013, the reporting was switched to fiscal year, so CY2013 only represents 9 months of data. Source: US Department of Agriculture - Red Books 2007-2016 (USDA, 2008b, 2009, 2011b, 2012a, 2013, 2014, 2015a, 2015b, 2017a, 2017b).

hydrochloride. With the subsequent approval of CEF CFA, the FDA again changed the tolerance for DFC to 0.4 ppm, which is the current tolerance level (US FDA, 2006a). These changes have resulted in the current meat withdrawals for CEF parenteral products.

Another change that occurred in residue monitoring programs was the implementation of MRM for verifying and quantifying residues in meat, replacing the 7-plate bio-assay (USDA, 2012b). However, due to the sophisticated extraction process required for CEF residues, CEF detection using an MRM process was not done prior to 2016. CEF was instead analyzed separately using the 7-plate bioassay for screening, a high-pressure liquid chromatography-ultraviolet detection (HPLC–UV) method to quantify the residue, and liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) for confirmation (Feng et al., 2014). Additionally, the KISTM test replaced an older in-plant screening test. Subsequently, it has been suggested that these new testing methodologies are more sensitive to CEF residues. However, published data suggests that the KISTM test has a sensitivity for CEF in kidney tissue of 4 ppm, nearly 10x higher than the tolerance (Jones et al., 2014). Therefore, the KISTM test is not very sensitive to CEF compared to the bovine tolerance. Published information on the 7-plate bioassay indicates that its sensitivity for quantifying residues was well below the tolerance of 0.4 ppm (USDA, 2008b). Furthermore, the new testing methodology has improved precision in quantifying residues (USDA, 2012b). Based on this information, changes in test sensitivity likely have little impact on increased CEF residue violations, but instead should have reduced the number of violative residues.

One of the major contributors to the increased number of violative CEF residues is marketing cull cattle earlier than their withdrawal date, which should be easily remedied by producer education programs. However, it is plausible that disease could be affecting drug pharmacokinetics (PK) in treated animals, thus increasing the time necessary for drug concentrations to reach tolerance levels.

Altered drug pharmacokinetics in diseased animals

Under the current drug approval process, drug manufacturers are required to undertake efficacy determination, toxicological safety testing, and residue depletion studies to determine withdrawal periods. In addition, microbiological safety testing is required for antimicrobials. Residue depletion studies are completed on healthy animals (US FDA, 2006b), which potentially have different drug PK than the diseased animals that veterinarians and their clients normally treat with these drugs. This may lengthen the depletion times needed for drug residues to fall below tolerance levels.

The primary PK parameters that impact the time it takes drugs to reach their tolerance levels in a given species are the dose administered, the rate of absorption, and the rate of excretion (Riviere et al., 1998). For most drugs, the rate of absorption is rapid, therefore elimination rate is dictated by the drug's V_D and clearance (CL). These two parameters are utilized to determine the drug's $T_{1/2}$. If either of these two parameters are altered significantly, depletion of the drug from the plasma can be substantially altered (Riviere, 2009a,b). For example, if $T_{1/2}$ is doubled, then it will take twice as long for the drug to be removed from the plasma. Depending on the physiologic ability to remove the drug at an organ of elimination (e.g., liver or kidneys), this may or may not closely resemble changes in clearance from the body and the resulting withdrawal intervals in food animals (Riviere et al., 1998).

There are several well characterized physiological factors that affect the PK of certain drugs, including age, gender, genetic variation, obesity, pregnancy in females, and circadian rhythms (Modric and Martinez, 2010). Furthermore, renal, hepatic, and cardiovascular disease are often associated with alterations in PK (Martinez and Modric, 2010). What is less understood is

the impact of inflammation or endotoxemia on PK of drugs, and the impact this may have on drug efficacy and withdrawal intervals.

Studies involving ceftiofur usage in the diseased bovine

Ceftiofur usage is widespread in dairy cattle (Zwald et al., 2004; Sawant et al., 2005; Schuler et al., 2017), yet there is minimal data comparing PK parameters of CEF between healthy and diseased cattle. In a summary article, Martinez and Modric (2010) conducted PubMed searches using the variables “pharmacokinetics” and “inflammation” from which they acquired 12 relevant hits for cattle. They received 14 relevant hits using the variables “pharmacokinetics” and “infection”. Of these 26, only 3 dealt with ceftiofur.

Erskine et al. (1995) compared drug concentrations in healthy cows versus cows experimentally challenged with IMM *E. coli*, using a non-conventional dosing strategy (3 mg/kg IV every 12 hours for 3 treatments). This resulted in all drug administration occurring within 24 hours. They reported no difference in peak concentrations (C_{\max}) of CEF in the serum of treated vs. healthy control cows. The assay used in this trial was a modified agar gel diffusion assay to determine bioactivity. They found higher serum concentrations of active CEF metabolites in the control group, suggesting that diseased cattle had increased distribution out of the plasma pool (Erskine et al., 1995).

Others have compared CEF concentrations using both HPLC and microbiological assay in feedlot cattle implanted with tissue chambers. Later, half of the chambers were inoculated with *Mannheimia haemolytica*. Both methodologies indicated that total CEF was significantly higher in infected than uninfected tissue chambers, even within a single animal. It should be noted that the described analytical procedure for determining CEF levels in plasma and other analytes, is to

convert all parent compound and metabolites to desfuroylceftiofur acetamide (DCA), and report these as CEF equivalents (Jaglan et al., 1990). Interestingly, the ratio of active CEF, determined with the microbiological assay, to total DCA was higher in the infected chamber than uninfected chamber. In this work, CEF was administered via the IV route and tissue chamber concentrations of CEF persisted longer than in plasma (Clarke et al., 1996). Later, a similar experiment was conducted using CEF CFA, which showed similar results, except that tissue chamber concentrations declined in parallel to those of plasma (Washburn et al., 2005). The investigators suggested that a higher concentration of total CEF (both protein bound and active) accumulates at the site of infection by passively moving through disrupted endothelial cell barriers and from the binding of CEF to acute phase proteins, such as α_1 -anti-trypsin, which rapidly move to sites of infection (Walker et al., 1994; Clarke et al., 1996; Washburn et al., 2005).

The work cited previously (Clarke et al., 1996; Washburn et al., 2005) should be interpreted cautiously, as tissue cages create an artificial, fluid-filled space surrounded by granulation tissue with porous vascular tissue (Davis et al., 2005). β -lactam antimicrobials have a low V_D , so they move into interstitial fluid from plasma but do not readily cross cell membranes (Papich and Riviere, 2009). Tissue cage environments creates physiological spaces that mimics interstitial fluid, for which water-soluble drugs like CEF can concentrate in higher concentrations than that of infected or inflamed tissues. These inflamed tissues normally are more cellular in nature with large amounts of fibrin, which will limit drug diffusion. Much of the drug in this area is protein-bound and would be biologically ineffective against infectious agents. However, the authors state that bound fractions of CEF will dissociate quickly in chemically reduced environments found in areas of inflammation (Clarke et al., 1996). Based on deficiencies in clinical relevance of tissue

chambers to interstitial fluid drug dynamics, investigational approaches that measure non-protein bound drug will provide data that is more clinically relevant (Davis et al., 2005).

North Carolina State researchers published a manuscript demonstrating altered PK and the need for increased milk withdrawal times for flunixin meglumine in cows affected with naturally occurring mastitis. In this trial, mastitic cows received both systemic FLU and CEF simultaneously (Kissell, et al., 2015). In plasma, FLU (Anderson, et al., 1990; Odensvik and Johansson, 1995) and metabolites of CEF (S. A. Brown, et al., 1991) are both reported to be >90% protein-bound in adult cattle, leading to a question as to whether co-administration of two highly protein-bound drugs may result in an interaction affecting protein binding of one or both drugs. Ceftiofur and flunixin are both weak acids, which primarily bind to albumin (Riviere, 2009a). In disease, albumin concentrations typically decrease to compensate for the body's need to increase production of acute phase proteins (Ceciliani et al., 2012), which should lead to a higher unbound fraction of the two drugs. As both CEF and flunixin are low extraction ratio drugs, an increase in the unbound fraction should reduce the total concentration of the drug in the circulation but not the free concentration (Toutain and Bousquet-Melou, 2002). Therefore, the prolonged milk withdrawal times for flunixin seen in this trial are likely due to changes in drug clearance due to decreased milk production or changes in drug metabolism associated with liver dysfunction due to disease (Kissell et al., 2015).

Studies involving ceftiofur usage in diseased animals in other species

Comparisons of CEF PK in diseased versus healthy animals have been completed in swine (Tantituvanont et al., 2009; Day et al., 2015; Sparks et al., 2016) and chickens (Amer et al., 1998). The swine trials included pigs experimentally infected with porcine reproductive and respiratory

syndrome virus (PRRSv) (Tantituvanont et al., 2009); pigs co-infected with PRRSv and *Streptococcus suis* (Day et al., 2015); and pigs infected with PRRSv and vaccinated (Sparks et al., 2017), all of which were compared to healthy control animals. In these trials, volume of distribution per fraction of the dose absorbed (V_D/F) and clearance per fraction of the dose absorbed (CL/F) were higher and DCA plasma concentrations were lower in diseased than healthy animals (Tantituvanont et al., 2009; Day et al., 2015; Sparks et al., 2017). In all three of the previously mentioned studies, changes in the V_D and CL are potentially confounded by differences in absolute bioavailability (F). None of the studies included an IV study to directly determine F , but Sparks et al. (2017) determined a relative F of 0.8, which decreased the differences in these two parameters in their group challenged with PRRSv. In the Sparks et al. (2017) study, the investigators demonstrated previous vaccination with a commercial PRRSv vaccine prior to viral challenge preserved PK parameters similar to that of the control animals.

In the poultry trial, one group of healthy chickens was exposed to aflatoxin in their diet while the control group was fed a diet devoid of aflatoxin. Both groups were treated with CEF via the IV, IM, or oral route. In chickens treated via the oral and IV route, serum concentrations of CEF were significantly lower and CEF was eliminated more quickly in birds exposed to aflatoxin (Amer et al., 1998).

Other studies investigating disease and altered pharmacokinetics

In a summary article evaluating the acute phase response to febrile disease, van Miert (1990) described the impacts on drug absorption, metabolism, and excretion. Diseases in which endotoxin is the inciting cause, gastric secretion and hunger sensations are absent in monogastric animals, which may change gastric pH and motility. Even though drugs are not directly absorbed

from the stomach, delayed gastric emptying may decrease intestinal absorption and drug solubility may be changed alterations in gastric pH.

In a trial by this research group, pigs were challenged with *Actinobacillus pleuropneumonia* and the effects on PK were evaluated. During the febrile state, orally administered oxytetracycline (OTC) took 4 times longer than control animals to achieve time to maximum concentration (T_{\max}) and C_{\max} was 50% of the concentration acquired in the control animals. Additionally, $T_{1/2}$ and AUC were increased in febrile animals, indicating that withdrawal times should be prolonged (van Miert, 1990).

In comparisons of absorption of ampicillin administered via the IM route, Groothuis et al. (1980) found absorption to be greater in febrile goats while shivering compared to non-febrile control goats. In contrast, less absorption and lower serum ampicillin concentration was noted in non-shivering, febrile calves compared to non-febrile controls (Groothuis et al., 1978).

Infection and inflammation potentially results in down regulation of drug transporters and hepatic and extra-hepatic cytochrome P450 metabolizing enzymes in the liver or intestinal epithelium. These alterations in cytochrome P450 activity usually decrease CL, but may increase CL. These changes are most important from a toxicology standpoint with low therapeutic index drugs, but also would be important from a drug residue standpoint in food-producing animals (Morgan, 2009).

Several studies suggest that inflammation of membranes surrounding joints or the membrane representing the blood milk barrier in the mammary gland are more porous when inflamed. As a result, this will allow more drug to penetrate the membrane (van Miert, 1990). However, three separate studies investigating drug diffusion across inflamed joint membranes have outcomes that are contrary to this suggestion. The first study investigated cephalixin diffusion

into suppurative synovial joints compared to normal joints on the same animal. Following cephapirin administration, drug concentrations were not significantly different between normal and infected joints (M. P. Brown et al., 1991). In a second contrary study, Cornell researchers described the effect of IV sodium penicillin G or IV OTC in calves following induced joint inflammation. After treatment, synovial fluid drug concentrations were not different in inflamed joints compared to healthy joints (Guard et al., 1989). Lastly, Shoaf et al. (1986) failed to show a difference in trimethoprim/sulfadiazine synovial fluid concentrations or plasma PK in neonatal calves. This demonstrates the need to characterize each drug and disease condition individually as it relates to drug PK.

Gips and Soback (1999) compared IV norfloxacin PK in milk and serum of cows with naturally occurring sub-clinical *Staph aureus* infections and cows with induced *E. coli* clinical mastitis. In both trials, norfloxacin concentrations and PK were compared to healthy, control cows. As a result, mean residence time (MRT) and $T_{1/2}$ were significantly lower in mastitis cows than the normal group. In cows with coliform clinical mastitis, CL increased as a function of disease severity and V_D was lower. In milk, area under the curve (AUC) was substantially higher than serum. Compared to normal cows and normal quarters in cows with mastitis, quarters with sub-clinical or clinical mastitis had lower milk concentrations and AUC. These findings were consistent with concentration predictions using the Henderson–Hasselbalch equation associated with mastitis induced milk pH changes (Gips and Soback, 1999).

In a study on the effects of induced *E. coli* mastitis on the pharmacodynamics and pharmacokinetics of carprofen, Dutch researchers demonstrated decreased systemic CL and a longer $T_{1/2}$ compared to the same animals under healthy conditions. In milk from healthy quarters,

carprofen was not detected, but in mastitic quarters it was detected for up to 45 hours after treatment (Lohuis et al., 1991).

Egyptian researchers compared the PK of marbofloxacin in cattle with *Mannheimia haemolytica* pneumonia to healthy animals following IV or IM administration. After IV administration, CL was lower while steady state V_D (V_{SS}) was not affected by disease, resulting in longer $T_{1/2}$, higher AUC, and higher MRT in the diseased animals compared to healthy animals. Intramuscular administration resulted in a higher C_{max} , higher AUC and MRT, and longer $T_{1/2}$. In the IM group, F and protein binding were not different between the two groups. As a result, the research indicated that the time above the minimum inhibitory concentration (MIC) was 12 hours in healthy cattle and 24 hours in diseased cattle (Ismail and El-Kattan, 2007).

In a clinical case report, Illinois researchers investigated the impact of theophylline as an ancillary therapy to provide bronchodilation in a group of 20 calves with naturally developing respiratory disease. Twenty control calves, also with respiratory disease, received a placebo in place of the theophylline. All calves also received spectinomycin and lincomycin in a 2:1 ratio by milligram weight, subcutaneously one time per day for 3 days. There were no differences in the clinical parameters monitored following initial therapy between treatment and placebo groups. Within 24 hours of the third treatment, 5 of the calves that received theophylline died, while no calves died in the control group. In the 5 theophylline treated calves that succumbed, mean trough theophylline concentrations were significantly higher than the 15 that survived. Additionally, the mean peak concentrations for the 5 dead calves was $> 20\mu\text{g/mL}$, which is the reported toxic concentration of theophylline in people. Interestingly, CL has been shown to be reduced in humans with concurrent febrile disease or administration of erythromycin or troleandomycin. The authors

of the manuscript speculated that the cause of death was theophylline toxicity associated with the concurrent respiratory disease and/or administration of antimicrobials (McKenna et al., 1989).

Kumar and Malik (1999) report that experimental infection with *Theileria annulata* and treatment with IM OTC resulted in T_{\max} , F , elimination $T_{1/2}$, AUC, and MRT values that were smaller than the same calves treated with IM OTC prior to the experimental inoculation. There was no difference in C_{\max} . The authors postulate that a shorter T_{\max} is likely the result of increased blood flow to peripheral muscle tissues as a result of the febrile response initiated in response to the infection, as previously shown by Groothuis et al. (1978, 1980). They further suggested that a more rapid elimination of the drug is likely due to increased renal blood flow and higher glomerular filtration rate associated with the febrile response, which has been described by van Miert (1990).

Taken together, these studies highlight the importance of investigating alterations in PK and potentially associated withdrawal times differences in diseased animals independently in each species, for each disease, and for each different drug. Additionally, physiological and genetic parameters must be considered. Veterinary practitioners need to consider whether it is prudent to extend withdrawal times with certain drugs and disease conditions.

Antimicrobial Resistance Associated with Cephalosporin Use

As dairy cattle are a dual-purpose breed, they provide food through milk and meat for human consumption. As there is potential for fecal contamination during the harvest process, both food sources could potentially deliver antimicrobial resistant bacteria to the consumer. Data gaps on the level of risk associated with the consumption of milk and dairy products are evident, particularly for extended spectrum beta-lactamase (ESBL) prevalence in pre-pasteurized milk,

whether from conventional or organic dairies (Horigan et al., 2016). A PubMed search using the keywords “ESBL” and “bulk tank milk” generated only four pieces of literature on the subject. From these, only three directly addressed EBSL prevalence in bulk tank milk (Geser et al., 2012; Sudarwanto et al., 2015; Odenthal et al., 2016). These manuscripts report ESBL prevalence in bulk tank milk to be non-existent in Switzerland (Geser et al., 2012) up to approximately 9% from *Enterobacteriaceae* in Indonesia (Sudarwanto et al., 2015) and Germany (Odenthal et al., 2016). However, when looking at the overall microbiota of bulk tank milk, coliforms represent a very small proportion of the total ecology. Additionally, several surveys studying prevalence of antimicrobial resistance (AMR) amongst mastitis pathogens have suggested that it is not increasing (Erksine et al., 2002; Makovec and Ruegg, 2003; Pol and Ruegg, 2007), despite the fact that mastitis therapy represents a substantial proportion of the total antimicrobial use on dairy farms (USDA, 2016a; Schuler et al., 2017). Therefore, the remainder of this literature review will focus on the impact of CEF therapy on the enteric microbial ecology and impact of delivery of AMR bacteria via meat from cull dairy cows.

Estimates of AMR among non-type specific (NTS) fecal isolates in dairy cattle herds are difficult to compare due to differences in trial design. One of the most consistent sources of data throughout the years on this topic has been the USDA NAHMS. In the 1996, 2002, and 2007 dairy herd surveys, USDA investigators collected 35-50 fecal samples per farm from approximately 100 dairy farms across 21 dairy states. The 2007 report summarized the data for *Salmonella* spp. isolates from the previous three surveys, including antimicrobial susceptibility data. To determine antimicrobial susceptibility, the gram-negative National Antimicrobial Resistance Monitoring System (NARMS) antimicrobial resistance plate was utilized. From the three surveys, 92.3%, 82.3%, and 96.6% of the *Salmonella* spp. isolates were pan-susceptible to all antimicrobials for

1996, 2002, and 2007, respectively (USDA, 2011b). In 2002, survey investigators also studied AMR in *E. coli* isolates and reported that 85.3% of the isolates were pan-susceptible (Lundin et al., 2008).

Policies and regulation regarding cephalosporin use

The use of antimicrobials in the dairy industry is commonplace (Zwald et al., 2004; Swant et al., 2005; Schuler et al., 2017). Concerns over the lack of prudent use has led the FDA to issue Guidance Policy 209 (US FDA, 2012a), which is a guidance document that describes the FDA's expectations for prudent antimicrobial use on farms. However, prudent use is still allusive on many dairy farms (Schuler et al., 2017). Lack of good antimicrobial stewardship may lead to loss of efficacy of the product against animal pathogens, but public health experts are mainly concerned about the development of AMR in commensal flora that may be spread via the food supply to humans (Doyle et al., 2013). In 2012, the US FDA implemented a restriction on the extralabel use of certain cephalosporin drugs in the major food producing species (cattle, swine, chickens, and turkeys). The prohibition did not include the first-generation cephalosporin, cephapirin, but prevents extralabel uses of all other cephalosporins labeled for the major food producing species for disease prevention or at unapproved doses, frequencies, durations, or routes of administration. Additionally, only cephalosporins specifically labeled for major food species can be used in these species. This prohibition was implemented due to the concerns about the development of AMR in food animals and the dissemination of the resistance determinants to humans through food consumption, thus risking public health (US FDA, 2012b). This action by the FDA represents the second time in which the agency has restricted certain uses of an approved antimicrobial over

concerns about the development of AMR, as fluoroquinolones also have extralabel restrictions for similar reasons to cephalosporins (US FDA, 1997).

In 2003, the first of two expert workshops was organized and conducted by the Food and Agriculture Organization of the United Nations (FAO), the World Organization for Animal Health (OIE), and the World Health Organization (WHO). From these workshops, three conclusions were developed: 1) the non-human use of antimicrobials was creating a clear human health consequence due to the development of resistant organisms; 2) human exposure to resistant bacteria from the consumption of animal food products was associated with the amount and pattern of non-human usage; and 3) the consequences were particularly severe when pathogens were resistant to antimicrobials critically important antimicrobials to humans. In 2005, the WHO classified 3rd, 4th, and 5th generation cephalosporins as critically important antimicrobials for the preservation of public health. In 2016, they changed their classification scheme slightly and now 3rd, 4th, and 5th generation cephalosporins are among the highest priority, critically important antimicrobials for the preservation of human health (WHO, 2017). During the same time period, the US FDA released Guidance for Industry #152 - Evaluating the Safety of Antimicrobial New Animal Drugs with Regard to Their Microbiological Effects on Bacteria of Human Health Concern. The purpose of this document was to recommend an approach to assess the effects of animal drugs on bacteria of human health significance. Like the WHO, they created a list of important antimicrobials for public health, classifying 3rd generation cephalosporins as critically important and 4th generation cephalosporins as highly important antimicrobials (US FDA, 2003).

Medically important cephalosporins listed by the WHO and the FDA include ceftriaxone, cefotaxime, ceftazidime, cefepime, and ceftaroline. Ceftiofur, a 3rd generation cephalosporin, is the only broad-spectrum cephalosporin approved for use in food animals in the US. It is the most

commonly utilized antimicrobial on dairy farms due to its broad spectrum of activity and short withdrawal periods (Zwald et al., 2004; Swant et al., 2005; Schuler et al., 2017). Resistance to ceftiofur results in cross-resistance to ceftriaxone and vice versa (US FDA, 2016b). Ceftriaxone is a 3rd generation cephalosporin often used in human medicine for the treatment of invasive enteric salmonellosis in children as fluoroquinolones are not approved for young children (Whichard et al., 2005; Doyle et al., 2013). Therefore, the use of ceftiofur in the veterinary industry is often scrutinized for its impact on cephalosporin AMR in humans, despite the fact that there is very limited evidence to support this accusation (Landers et al., 2012; Doyle et al., 2013).

Mechanisms of antimicrobial resistance against cephalosporins

For bacteria to develop resistance against cephalosporins and other β -lactams, they must employ at least one of the following mechanisms: 1) develop mutations that alter the structure of their penicillin-binding proteins; 2) alter cell wall permeability through disruption of porin proteins or upregulation of efflux pumps; and/or 3) production of β -lactamase enzymes which hydrolyze the β -lactam ring of the antimicrobial (Seiffert et al., 2013). Extended spectrum β -lactamases are the primary cause of AMR in *Enterobacteriaceae* isolates on a worldwide basis (Bonnet, 2004; Zhao and Hu, 2013). These β -lactamases are encoded by genetics found on chromosomes or on plasmids.

Currently there are >1000 β -lactamase enzymes described in the literature (Bush and Fisher, 2011), which can be in one 34 families (NCBI, 2017). Classification of β -lactamases occurs through placement into one of four Ambler class, which is a molecular classification system. Additionally, they are classified into one of four functional groups (Bush groups 1-4) (Bush and Jacoby, 2009). Ambler classes A, C, & D are serine enzymes, while class B are metallo enzymes.

Resistance to 3rd generation cephalosporins is primarily mediated by AmpC β -lactamases from class C or ESBL's, such as TEM, SHV, and CTX-M from class A (Bradford, 2001; Paterson and Bonomo, 2005; Bush and Jacoby, 2009). Over the last 20 years, plasmid-mediated AmpC-like (*bla*_{CMY}) and *bla*_{CTX-M} genes appear to be the most important for development of AMR in *Enterobacteriaceae* against cephalosporins in food producing animals (Li et al., 2007).

AmpC β -lactamases

AmpC β -lactamases can be found on chromosomes or plasmids. Chromosomal AmpC β -lactamases are commonly found in enteric bacteria, with their expression being constitutive or inducible. Inducible forms found in enteric bacteria often are controlled by several regulators. Novel mutations have been shown to increase the spectrum of chromosomal AmpC β -lactamases to include 4th generation cephalosporins (Mammeri et al., 2006).

*Bla*_{CMY} genes are plasmid-mediated AmpC-like genes. They were described in a review article by Li et al. (2007) as the most widespread plasmid-mediated β -lactamase genes found in *E. coli* and *Salmonella* spp. isolates of animal origin. In the US, 3rd generation cephalosporin resistance in *E. coli* and *Salmonella enterica* isolates from food animals from 2000 – 2010 was most commonly associated with the plasmid-borne *bla*_{CMY-2} gene (Daniels et al., 2009; Davis et al., 2015). *Bla*_{CMY} β -lactamases have a wide spectrum of activity against a variety of cephalosporins, including cephamycins (cefoxitin) and oxyimino-cephalosporins (e. g., cefotaxime, ceftriaxone, and ceftazidime). Plasmid-mediated *bla*_{CMY} β -lactamases are not inhibited by clavulanate but are inhibited by carbapenems. However, *bla*_{CMY} β -lactamases are not considered ESBL's. (Bradford, 2001; Li et al., 2007). Plasmids encoding *bla*_{CMY} resistance usually carry multiple AMR genes and are associated with transposons or integrons, allowing for this plasmid

to be easily disseminated within enteric bacteria and other genera (Li et al., 2007). Surveys of *Salmonella* isolates from several different outbreaks indicate that resistance genes against ampicillin, chloramphenicol, streptomycin, sulfisoxazole, and tetracycline (ACSSuT phenotype) have been found on *bla*_{CMY} plasmids (Winokur et al., 2000; Rankin et al., 2002; Doyle et al., 2013). These resistance determinants have been associated with the Inc A/C plasmid, which is highly troublesome as this plasmid has the ability to acquire large numbers of resistance determinants and is easily able to circulate through wide variety of bacterial hosts, both commensals and pathogens (Doyle et al., 2013).

Plasmid-mediated bla*_{CTX-M} *ESBL's

While ESBL's can be β -lactamase derivatives of *bla*_{TEM}, *bla*_{SHV}, or *bla*_{OXA}; currently, the dominant worldwide genetic profile of ESBL's is that of *bla*_{CTX-M} (Davis et al., 2015). These enzymes belong to Ambler class A or D and the Bush group 2be or 2d (Li et al., 2007). CTX-M β -lactamase enzymes were first described in 1986 from a canine fecal *E. coli* isolate and have since been described worldwide (Bonnet, 2004). CTX-M genes are believed to have arisen from chromosomal genes of *Kluyvera* spp. (Canton et al., 2012). Dissemination of *bla*_{CTX-M} genes are described as being plasmid associated, with incompatibility group I1, F, or N commonly found as the vehicle for dissemination (Mollenkopf et al., 2012).

This enzyme is named due to its efficiency at hydrolyzing cefotaxime (Li et al., 2007). CTX-M β -lactamase enzymes typically result in high levels of resistance in *E. coli* against ampicillin, amoxicillin, carbenicillin, ticarcillin, piperacillin, cephalothin, cephaloridine, cefuroxime, cefotaxime, and ceftriaxone; but maintain susceptibility to ceftazidime and carbapenems. These enzymes have a variable level of resistance against cefepime, ceftazidime, and ceftazidime,

although it appears that variants of the CTX-M enzyme are emerging that improves activity against ceftazidime. Minimum inhibitory concentrations (MIC's) against aztreonam are also high. Susceptibility to β -lactamase inhibitors is variable. Combinations of clavulanate and either amoxicillin or ticarcillin usually demonstrate effectiveness or low-level resistance against bacteria expressing the CTX-M gene. Piperacillin/tazobactam combinations, as well as cefotaxime combined with clavulanate or tazobactam, are effective against CTX-M harboring bacteria (Bonnet, 2004; Wittum et al., 2010). These genes are predominantly responsible for the ESBL phenotype in *E. coli* and *Klebsiella* spp. in humans worldwide (Bonnet, 2004; Zhao and Hu, 2013). Plasmids encoding for *bla*_{CTX-M} often harbor the *bla*_{TEM} gene, as well as genes encoding for resistance to aminoglycosides, chloramphenicol, sulfonamides, trimethoprim, and tetracyclines (Li et al., 2007; Seiffert et al., 2013).

In the mid-2000's, *bla*_{CTX-M} was beginning to be described from livestock in Europe and Asia (Watson et al., 2012). The first US description of *E. coli* with *bla*_{CTX-M} in dairy cattle was in 2009 from OH (Wittum et al., 2010). It is plausible that the recent description of *bla*_{CTX-M} is only occurring because scientist did not specifically look for *bla*_{CTX-M} genes in resistant isolates in early research. However, in a retrospective study, Washington State University researchers re-examined resistant *E. coli* isolates from previous studies to scan for additional resistance determinants. Eighty-nine percent of isolates obtained prior to 2011 were carrying the *bla*_{CMY-2} gene and none carried *bla*_{CTX-M} genes. From isolates obtained in 2011, there was a combination of *bla*_{CMY} and *bla*_{CTX-M} resistance genetics prevalent in isolates from Washington state dairies (Davis et al., 2015).

Current status of antimicrobial resistance from cattle at processing

The National Antimicrobial Resistance Monitoring System (NARMS) is a collaborative effort between the US FDA; US Centers for Disease Control (CDC); USDA; state and local health departments; and universities to monitor AMR in humans, retail meats, and food producing animals. Additionally, NARMS scientists conduct epidemiological investigations about risk factors and clinical outcomes for certain infections and investigate molecular mechanisms for development of resistance. Prevalence of AMR in *Salmonella* and *Campylobacter* are monitored, as they are major causes of human food-borne bacterial illness. In addition, NARMS scientists also monitor *E. coli* and *Enterococcus* from retail meat and cecal samples as indicators of resistance development in gram-negative and gram-positive bacteria, respectively. The most recent data available from NARMS is from 2014 (US FDA, 2016b).

The following NARMS data is summarized by production classes. Dairy samples are defined as dairy cull cows; while beef samples are defined as samples from steers, heifers, and cull beef cows. NARMS also includes sample data from the USDA Food Safety and Inspection Service's (FSIS) Pathogen Reduction/Hazard Analysis and Critical Control Point (PR/HAACP) sampling program, in which they swab all classes of cattle carcasses at the end of processing.

In 2014, the first evaluations of cecal samples were published, as this testing was only implemented in 2013. 10.1% of *Salmonella* isolates from cecal samples of dairy cattle were found to be multi-drug resistant (MDR), with MDR defined as being resistant to three or more antimicrobial classes. Beef samples had an MDR level in *Salmonella* of 5.8%, while USDA FSIS PR/HAACP testing revealed that 16.6% of the cattle carcasses had MDR *Salmonella* present. Of the *E. coli* cecal isolates, 12% of dairy cattle isolates were MDR.

Ceftriaxone resistance was reported at 7.6%, 1%, and 5.5% for PR/HAACP, beef, and dairy *Salmonella* isolates, respectively. The level reported in PR/HAACP testing is the lowest reported since 1999. Additionally, the level of isolates with MDR was also the lowest in cattle since 1999. However, *Salmonella* serotype Newport had a 53% level of ceftriaxone resistance from cattle isolates. Resistance among *Salmonella* serotype Dublin also continues to be a concern, with 29% of the isolates exhibiting ceftriaxone resistance. This was down substantially from 2013, when approximately 85% of the isolates were resistant. The FDA's cephalosporin restriction was cited as one potential possibility for the decline in ceftriaxone resistance seen in 2014.

Resistance determinants for ceftriaxone resistance in *Salmonella* and *E. coli* isolates in NARMS reports have traditionally been related to the *bla*_{CMY-2} gene. However, recent data suggests the *bla*_{CTX-M} is increasing. 2014 cattle isolates from PR/HAACP testing of non-typhoid *Salmonella* exhibiting the ACSSuT phenotype were at 7.1%. This is down substantially from 2009, when the prevalence was 67%. However, in dairy cattle cecal samples, 6 of 10 samples exhibited the ACSSuT phenotype (US FDA, 2016b).

Ceftiofur usage and the development of antimicrobial resistance

One of the biggest challenges proving associations between ceftiofur usage and resistance among fecal NTS *E. coli* at the animal level is a failure to access individual animal records and quantitate antimicrobial usage (Horigan et al., 2016). There has been a plethora of studies looking at herd level associations between various antimicrobials and the presence of important resistance genes, but few have specifically accounted for individual antimicrobial usage and resistance patterns at the animal level

One of the more highly cited dairy surveys is work done by Tragesser et al. (2006). In a survey of 18 Ohio dairy herds, this research group collected 1266 fecal samples to determine the level of ceftriaxone resistance and attempt to correlate this resistance with CEF usage on the source farm. This research group found that at least one cow exhibited ceftriaxone resistance in 67% of the surveyed herds. Overall, 34.4% of the *E. coli* isolates had resistance to ceftriaxone. In herds that reported usage of CEF within the previous 6 months, the mean ceftriaxone resistance was 40% (range 0-97%), while the mean resistance in herds that reported no ceftiofur usage was 9% (range 0-34%). The presence of the *bla*_{CMY-2} gene was found in 83% of the resistant isolates. Eighty-one of the ceftriaxone resistant isolates were further tested against a standard panel of antimicrobials, where resistance was confirmed for amoxicillin, clavulanic acid, ampicillin, cephalothin, ceftiofur, streptomycin, sulfamethoxazole, and tetracycline. These researchers determined that there was a herd level association between the use of CEF and ceftriaxone resistance, but not a cow level association. However, the associated use on the participating farms could be as long as 6 months prior to the time of sampling and did not identify individual animals that were treated with CEF (Tragesser et al., 2006).

The following year, Lowrance et al. (2007) published similar results after following the treatment of beef feedlot steers with various dosage regimens of CEF CFA compared to commingled, non-treated control animals. In this work, they found 68.4% of fecal *E. coli* isolates harbored phenotypic resistance to at least one antimicrobial. Following CCFA therapy, treated animals experienced transient increases in CEF resistant *E. coli* populations that returned to pre-treatment levels in approximately 2 weeks. As with the Tragesser et al. (2006) study, this research group determined a high level of correlation with CEF resistance and co-resistance against ampicillin, chloramphenicol, streptomycin, sulfisoxazole, and tetracycline (ACSSuT phenotype).

Additionally, they described an association between CEF usage and reduced susceptibility to ceftriaxone. While no direct testing of genetic determinants of resistance were undertaken in this trial, the authors hypothesized that resistance to CEF and ceftriaxone was likely due to the *bla_{CMY}* gene.

In a calf study evaluating ceftriaxone resistance, three dairy breed calves were injected with a 5-day course of CEF hydrochloride at therapeutic dosages and three were left as untreated controls. The two treatment groups were housed separately, so horizontal spread of resistant bacteria from treated to control calves was not possible. Total fecal bacterial counts and total ceftriaxone resistant fecal bacteria counts were compared between the two groups. Ceftriaxone growth was assessed on antimicrobial impregnated agar plates containing 16, 64, and 128 µg/mL of ceftriaxone. It should be noted that the CLSI breakpoint level of ≥ 64 µg/mL of ceftriaxone for determining resistance used in this study is higher than the current breakpoint of ≥ 4 µg/mL (CLSI, 2010; CLSI, 2011). The calves were followed for 12 days following CEF treatment. Following CEF therapy, there was a 0.5-1 log reduction in total bacteria count, which persisted throughout the remainder of the study. However, 5 days following the last CEF treatment, the two groups had similar total bacteria counts. There was a significantly higher number of resistant bacteria in the CEF treated group on agar plates containing 16 and 64 µg/mL levels of ceftriaxone from the day of treatment throughout the remainder of the observation period. Additionally, the percent ceftriaxone resistant bacteria as a percentage of the total were determined to be significantly elevated on days 0, 1, 2, 3, and 17. It is interesting to note that ceftriaxone resistant bacteria were already significantly different in the treatment group at the time of the first treatment, whereas one-day prior to CEF treatment they were not different. The resistance genes found in isolates were *bla_{CMY-2}*. The authors concluded that CEF treatment in young calves can temporarily change the

bacterial flora in a calf's intestine to favor the growth of ceftriaxone resistant bacteria (Jiang et al., 2006).

The trials conducted by Jiang et al. (2006) and Lowrance et al. (2007) were not designed to determine horizontal transmission of resistant bacteria genes. Therefore, Singer et al. (2008) designed a trial to mix treated and untreated groups in the same pens to assess horizontal transfer of resistance. Five lactating dairy cows received a five-day course of CEF hydrochloride at therapeutic dosages. The control group of five matched, untreated cows were penned in the same pen as the treated cows. From two days following the initial treatment through three days post-treatment, the treatment group had a significantly reduced total *E. coli* count and significantly elevated antimicrobial resistance index. Antimicrobial resistance index was used by the study authors as a quantitative measure of antimicrobial resistance in a bacterial population. The total *E. coli* count and antimicrobial resistance index remained unchanged in the control cows throughout the study. The *bla*_{CMY-2} gene was detected in the majority of the resistant fecal isolates of treated animals through day 6 and at no point afterward. No resistance genes were detected in control animals. The authors employed community DNA polymerase chain reaction (PCR) analysis for this study, so were unable to determine the genera of the source bacteria of the *bla*_{CMY-2} gene (Singer et al., 2008).

Prior to initiation of the above study (Singer et al., 2008), the investigators were able to detect resistant *E. coli* containing the *bla*_{CMY-2} gene from calves in the herd that had no prior antimicrobial treatment. They were also able to occasionally detect these resistance genes in untreated adult animals in the herd. The authors hypothesized that the presence of these resistant strains may be affected by bacterial fitness in relationship to other environmental factors. They summarized that these findings, along with the results of this trial, indicate that resistance genes

were circulating in the herd over time, but CEF treatment increased the probability of isolating resistant strains. The authors concluded the manuscript by stating “...this study emphasizes that the finding of resistant isolates following antimicrobial treatment is not sufficient to estimate the strength of a selection pressure, nor is it sufficient to demonstrate a causal link between antimicrobial use and the emergence or amplification of resistance” (Singer et al., 2008, p. 6961).

In a later study by the same research group, fecal samples from the previous study (Singer et al., 2008) were utilized to perform community DNA profiling to seek out the *bla*_{CMY-2} gene using culture independent techniques. From this work, they confirmed their previous conclusions that treated animals had a transient increase in resistance genes in the bacterial community, which returned to pre-treatment levels by day 6. They also detected slight increases in resistant DNA in the five untreated cows, suggesting that resistant genes were being horizontally transmitted amongst animals. The bacterial species harboring the resistant genes in the control group were likely not *E. coli* as the previous study detected no increase in resistant *E. coli* (Boyer and Singer, 2012).

To understand the effects of CEF treatment on *bla*_{CMY-2} plasmid transfer, Daniels et al. (2009) inoculated calves with bacterial flora containing resistance plasmids and recipient bacterial flora to CEF treated and untreated controls. The hypothesis of the study was that movement of resistance plasmids would occur from donor bacteria to recipient bacteria not containing the plasmids and the CEF treatment would heighten the level of transmission. The results showed that transmission of plasmids between the donor and recipient bacteria did occur, but at a low frequency. Additionally, CEF treatment did not enhance the development of resistant strains (Daniels et al., 2009).

In the cited studies in this literature review, the CEF treated animals were healthy with the exception of Singer et al. (2008), where animals were positive for *Leptospira borgpetersenii* serovar Hardjo-bovis. This spirochete causes sub-clinical infections and purportedly was responsible for economically significant losses due to early embryonic death in dairy cattle. However, the animals did not appear clinically ill. The only other study found in the literature that dealt with clinically ill animals involved treatments for clinical metritis or foot rot. Cattle on two separate NY dairy farms with these ailments could be treated with either CEF or penicillin (Mann et al., 2011). Like the previously mentioned studies, a reduction in total *E. coli* colony forming units (cfu) counts was noted following CEF treatment. The authors also reported a significant association between CEF resistance from *E. coli* isolates and concurrent ampicillin and tetracycline resistance. Interpretation of levels of *E. coli* CEF resistance from this manuscript is confounded by data presentation, as the authors combined all collection days into one pool. The authors reported that a significant reduction in CEF susceptibility was not determined following treatment. In this study, there was no healthy group of animals treated with CEF to serve as a positive control group (Mann et al., 2011).

One consistent pattern amongst the studies in this literature review is the transient reduction in fecal *E. coli* and in some cases increased proportions of resistant *E. coli* strains. These findings have also been reported with administration of florfenicol in feedlot cattle (Berge et al., 2005), tetracycline in feedlot cattle (Stabler et al., 1982), and penicillin in milk fed to Holstein calves (Langford et al., 2003).

Several studies have looked for risk factors for presence of resistant *E. coli* associated with the *bla*_{CTX-M} or *bla*_{CMY} genes. Risk factors determined to be influential for an increased herd prevalence were: use of 3rd or 4th generation cephalosporin antimicrobials (Snow et al., 2012;

Gonggrijp et al., 2016); younger age of the studied animal (Snow et al., 2012; Geser et al., 2012; Schmid et al., 2013); not maintaining a closed herd or not quarantining new arrivals (Snow et al., 2012; Schmid et al., 2013; Davis et al., 2015); recent addition of animals (Schmid et al., 2013; Davis et al., 2015); larger herd size (Davis et al., 2015; Hille et al., 2017); higher herd level milk production (Heider et al., 2009); treatment of all clinical mastitis cases with antimicrobials (Gonggrijp et al., 2016); use of dry cow teat sealant on selected cows versus all cows (Gonggrijp et al., 2016); not using a dry cow teat sealant on any cows vs all cows (Gonggrijp et al., 2016); using automated manure scraping (Snow et al., 2012; Gonggrijp et al., 2016); lack of fly control (Davis et al., 2015); increasing frequency of antimicrobial use in calves (Gonggrijp et al., 2016); less frequent bedding calf housing (Davis et al., 2015); and reduced frequency of cleaning calf rearing equipment (Snow et al., 2012; Davis et al., 2015). Interestingly, total antimicrobial use had no relationship with risk of finding resistant isolates in several studies (Snow et al., 2012; Davis et al., 2015; Gonggrijp et al., 2016).

In one study, concurrent isolation of *Salmonella* spp. from fecal samples was correlated with isolating *E. coli* with reduced susceptibility to ceftriaxone. However, the *Salmonella* spp. isolates did not have reduced ceftriaxone resistance (Heider et al., 2009). Daniels et al., (2009) reported that farms with a higher prevalence of *Salmonella* isolation utilized more CEF. However, it is unlikely that this association is having an impact in the Heider et al. (2009) study, as higher CEF usage should lead to elevated levels of ceftriaxone resistance in *Salmonella* spp. isolates.

In contrast to studies that found associations between cephalosporin use and presence of resistant strains of *E. coli*, several studies have not found an association between CEF use and isolation of β -lactamase producing CEF resistant *E. coli* (Daniels et al., 2009; Heider et al., 2009; Mollenkopf et al., 2012; Ohnishi et al., 2013; Sato et al., 2014; Davis et al., 2015).

Two manuscripts have been recently published that have looked at prevalence of resistant *E. coli* associated with ESBL or AmpC genes on organic farms (Dolejska et al., 2011; Santman-Berends et al., 2017). These studies were able to make comparisons to conventional farms using similar study designs. The first reported an animal level prevalence of resistance of 39% in conventional herds compared to <1% in organic herds. From these results, the authors suggested a possible link between cephalosporin use and resistance development (Dolejska et al., 2011). In the second study, Dutch researchers reported a 13% herd prevalence in organic herds (Santman-Berends et al., 2017) compared to 41% in conventional herds (Gonggrijp et al., 2016). It should be noted that the latter organic study (Santman-Berends et al., 2017) followed organic definitions of the European registration program, which allows up to three antimicrobial treatments per cow per year, as long as the treatments are prescribed by a veterinarian. Like the conventional herds, there was no association between total antimicrobial use and prevalence of herds with resistant isolates. However, unlike the conventional herd study, there was no association between total doses of 3rd and 4th generation cephalosporins used and prevalence of herds with resistant isolates. There was an association between positive herds and vicinity to swine farms; occasionally treating clinical mastitis with parenteral antimicrobials; and feeding milk replacer instead of whole milk to calves (Santman-Berends et al., 2017). While not the intention of the study design, the aforementioned study by Daniels et al. (2009) contained 3 organic herds and 39 conventional herds. The only farm in their study that reported no resistance to ceftazidime was one of the organic farms. In this study, ceftazidime resistance was used as an initial marker for carriage of the *bla*_{CMY} gene. Determination of the actual association between ceftazidime resistance and carriage of the *bla*_{CMY-2} gene was done by PCR, with 89% of the ceftazidime resistant isolates confirmed to be carrying the *bla*_{CMY-2} gene. Interestingly, the other two organic farms had prevalence frequencies of ceftazidime resistance

that were similar to the conventional farms. This indicates that antimicrobial usage, including CEF, is not necessary for the preservation of *E. coli* harboring the *bla*_{CMY-2} gene in the microenvironment (Daniels et al., 2009).

Other risk factors associated with development of antimicrobial resistance

It is clear that the use of antimicrobials will result in the development of AMR fecal NTS *E. coli*, at least transiently (Jiang et al., 2006; Lowrance et al., 2007; Singer et al., 2008). However, interactions with other environmental exposures may be associated with the development of AMR, without prior antimicrobial use. Examples include membrane efflux pumps that may be upregulated due to the presence of substrates, such as heavy metals or disinfectants (Summers, 2002). Another example would be resistance genes that are housed on molecular structures that evolve in a population of bacteria. For instance, the genes for the siderophore aerobactin have been shown to be present on plasmids that also contain AMR genes. Low environmental iron conditions that favor the expression of bacteria with the aerobactin siderophore could also facilitate AMR in these bacteria (Delgado-Iribarren et al., 1987). In another example, Washington state investigators showed a positive relationship between feeding milk to calves containing a vitamin D supplement and the proliferation of *E. coli* with resistance to streptomycin, sulfadiazine, and tetracycline (SSuT strains). The investigators postulated that the presence of the resistance genes in the feces of supplemented calves was likely due to factors that provided either a direct and/or indirect competitive advantage to the bacteria in the presence of the supplement (Khachatryan et al., 2006). These findings indicate that epidemiological investigations into the presence of AMR need to fully investigate the complex mechanisms that may be involved in the exhibition of resistance genes, instead of simply assuming a cause and effect relationship of antimicrobial usage.

Summary - antimicrobial usage, resistance development, and spread to humans

On a worldwide stage, the incidence of human infections caused by *E. coli* resistant to 3rd generation cephalosporins is increasing (de Been et al., 2014; Thaden et al., 2016). Public health experts fear that the use of antimicrobials in farm animals, especially CEF, is a major risk factor associated with the development of disease from ESBL *Enterobacteriaceae* (Doyle et al., 2013; Landers et al., 2012). Recent work utilizing whole genome sequencing is proving that previous work done using lower resolution methods have incorrectly characterized resistant *E. coli* isolates from human and poultry. These isolates from humans and poultry had previously been described as indistinguishable, but high-resolution sequencing has determined that the isolates are unrelated and that plasmids from distinctly different reservoirs are the cause of the AMR (de Been et al., 2014).

Characterizing CEF use in dairy herds and trying to associate this use with the development of resistance must be done with some caution due to the complex interactions that are taking place (Landers et al., 2012). One of the biggest challenges in herd-based risk assessments regarding the impact of antimicrobial use is confirming the actual antimicrobial consumption. As is evident from studies where known animals were treated (Lowrance et al., 2007; Jiang et al., 2006; Singer et al., 2008), treatment with the CEF results in a transient decrease in total *E. coli* and an increased proportion of resistant strains. However, this is not unique to CEF, as data shows similar patterns when other antimicrobials are administered. Additionally, AMR has routinely been demonstrated in bacteria in which no antimicrobial use has occurred, suggesting a role of AMR genes in organism fitness. Previously published risk assessments looking at the relationships between the use of a single antimicrobial in livestock and adverse human effects have been shown to have data

gaps that call into question the estimation of risk (McEwen, 2012). Additionally, models studying AMR transmission between animals and humans suggest that reducing antimicrobial use in animals will have little effect on AMR development in humans (van Bunnik and Woolhouse, 2017).

In the future, the use of advanced diagnostic techniques, such as high-resolution sequencing, and well-designed risk assessments are paramount for a more thorough understanding of antimicrobial usage in food producing animals and subsequent impacts on public health. Additionally, there is little information about the impact of antimicrobials as they are introduced into the bio-system following animal use and the impact on bacterial ecology on plants and water reservoirs. There are also suggestions that models could be created utilizing pharmacological, microbiological, and animal production components to implement a post-treatment resistance reversion period, similar to withdrawal times to reduce the risk of spread of AMR mechanisms (Volkova et al., 2016). In the meantime, the implementation of effective preventative medicine programs and prudent antimicrobial use policies on farms will help relieve the need to use antimicrobials in food animals and will have some impact on development of AMR in farm animals.

Conclusion

Ceftiofur usage in the dairy industry is commonplace (Zwald et al., 2004; Swant et al., 2005; Schuler et al., 2017) and violative antimicrobial residues associated with CEF usage have been the most common violative residue identified in cull dairy cattle since 2013 (USDA, 2015 a,b; USDA, 2017 a,b). The potential causes for said violative residues are many, but one potential source that has not been investigated is the impact of sickness on CEF pharmacokinetics, resulting

in prolonged drug elimination and the need to extend withdrawal periods prior to slaughter. The first objective of this dissertation project was to investigate the impact of moderate or severe clinical mastitis on the pharmacokinetics of CEF versus PK in healthy animals (Chapter 2 and 4). The second objective was to determine if the co-administration of two commonly used veterinary drugs in the treatment of clinical mastitis impacted the PK of each other (Chapter 3). The third objective was to determine the impact of moderate or severe clinical mastitis on the clearance of CEF from tissues of regulatory interest (Chapter 4).

Antimicrobial resistance is an increasing public health concern on a worldwide basis. Third generation cephalosporins have been designated as medically important antimicrobials by the WHO (WHO, 2017) and the US FDA (US FDA, 2003). In the veterinary industry, CEF usage is a major concern as it is closely related to ceftriaxone, an antimicrobial commonly used in human medicine for the treatment of invasive enteric Salmonellosis in children (Whichard et al., 2005; Doyle et al., 2013). This has prompted the US FDA to implement a prohibition against extra-label CEF usage in the major food producing species (US FDA, 2012b). Several manuscripts have investigated AMR in NTS fecal *E. coli* and describes this to be transient in nature (Jiang et al., 2006; Lowrance et al., 2007; Singer et al., 2008). However, there is little to no literature as to whether disease has any impact on the transient nature of AMR in NTS fecal *E. coli*. The transient nature of NTS *E. coli* has led at least one research group to suggest the implementation of withholding periods to minimize human exposure to resistant organisms, similar to those imposed to prevent drug residues in meat and milk (Volkova et al., 2016). These withholding periods would be established based on several variables, including pharmacokinetics of the drug administered. However, little information is available on persistence of AMR in NTS fecal organisms in ill versus healthy animals. The final objective is to investigate AMR patterns: 1) of *E. coli* isolated from

clinical mastitis cases; 2) of *E. coli* from systemic infections associated with clinical mastitis cases; and 3) of NTS fecal *E. coli* recovered from cattle experiencing clinical mastitis and healthy cattle treated with CEF (Chapter5).

CHAPTER 2.

**ALTERED PLASMA PHARMACOKINETICS OF CEFTIOFUR
HYDROCHLORIDE IN COWS AFFECTED WITH SEVERE CLINICAL
MASTITIS**

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Abstract

Mastitis is a frequent problem among dairy cows, reducing milk yield and increasing cull rates. Systemic therapy with the cephalosporin antimicrobial ceftiofur hydrochloride (CEF) may improve therapeutic outcomes, but the incidence of CEF violative residues has increased annually since 2011. One potential explanation is that disease status may alter the pharmacokinetics (PK) of CEF. To test this hypothesis, we compared the plasma PK of CEF in healthy cows with those with severe endotoxic mastitis.

Eight cows with naturally occurring mastitis and eight clinically healthy cows were treated with 2.2 mg of CEF/kg of body weight once daily for five days via the intramuscular route. Blood was collected at 0, 0.33, 0.67, 1, 1.5, 2, 3, 4, 8, 16, and 24 hours after the first CEF administration and every eight hours thereafter until 120 hours after the final dose. Plasma samples were analyzed for CEF concentrations using liquid chromatography coupled with mass spectrometry. With the exception of time 0, CEF was detected at all time points. The disease group had a significantly higher plasma CEF concentration at T=3 hours after the first injection and a significantly lower plasma concentration from 40-152 hours following the first injection, with the exception of the T=64 hour time point. Data following the first injection (Time 0-24 hour) were fit to a single dose, non-compartmental PK model. This model indicated that the disease group had a shorter plasma half-life. A multi-dose, non-compartmental model was used to determine steady state PK. Compared to control cows, the disease group had an initially higher peak concentration, a higher volume of distribution, and higher drug clearance rates. The disease group also had a lower area under the curve per dosing interval, steady state concentration maximum, and dose-adjusted peak steady state concentration. All other PK parameters were not different between the two groups. Altered PK, as suggested by this trial, may contribute to an increased risk for the development of a violative residue in meat. Further research is needed to more completely characterize drug distribution in diseased cattle and to study the effect of co-administration of other drugs on drug distribution.

Key words: bovine mastitis, ceftiofur, pharmacokinetics, drug residues

Introduction

Mastitis is the most common reason for therapeutic antimicrobial use in dairy cattle, according to the 2007 USDA National Animal Health Monitoring Survey. This survey of the dairy industry in the United States indicated that 18.2% of respondents' cows had been treated for mastitis during the previous 12 months (USDA, 2008a). Coliforms are the most common bacterial group causing clinical mastitis, and these infections generally result in more severe infections, with decreased survivability, than other pathogens (Oliveira et al., 2013; Wenz et al., 1998; Erskine et al., 2002).

Reasons for decreased survivability following mastitis include the development of endotoxic conditions with severe intramammary (IMM) tissue damage and, the potential development of secondary disease. Secondary bacteremia develops in 45% of cows with severe mastitis (Wenz et al., 2001a). Systemic ceftiofur (CEF) treatment can potentially address severe mastitis and secondary infections. When treated with CEF, fewer cows were sold or died as a result of severe coliform mastitis as compared to cows that were not treated (Erskine et al., 2002). CEF is a third-generation cephalosporin that is appropriate for use against coliform infections if effective concentrations can be achieved and maintained at the infection site. As a result, systemic treatment with CEF, with or without IMM therapy, has been included in many veterinary treatment protocols for moderate or severe clinical mastitis.

Over the past several years, concerns about veterinary drug use have increased, especially around the impact on antimicrobial efficacy in humans and the presence of drug residues in milk and meat. Since FY2011, violative CEF residues in culled dairy cows from inspector-generated samples have increased more than 5-fold. (USDA, 2013; USDA,

2014a). In 2012, the US Food and Drug Administration (FDA) issued a prohibition against extra-label drug use of cephalosporins in bovines (US FDA, 2012). Given the prohibition of a drug so important to the dairy industry, there is clearly a significant regulatory concern about the increase in violative residues. Although the cause of increasing CEF residues is likely multi-factorial, one potential explanation may be altered pharmacokinetics (PK) and residue depletion of CEF in diseased dairy cattle.

During the drug approval process, sponsoring companies must present the FDA Center for Veterinary Medicine (CVM) with toxicological and residue depletion studies. Based on these data, the FDA CVM establishes withdrawal periods for meat and milk, if approved for lactating dairy cattle. However, these studies are performed on healthy animals, not animals suffering from infectious diseases (US FDA, 2006a). Because antimicrobials are not intended for healthy animals, data on drug metabolism in diseased animals would provide veterinarians with evidence to more accurately prescribe veterinary drugs and to better predict residue depletion in these diseased animals. In turn, this could improve treatment efficacy and reduce the risk for violative residues in marketed animals. However, there are few data from cattle available in the veterinary literature addressing this topic. The objective of this study was to compare the plasma PK of CEF between healthy dairy cattle and those afflicted with severe clinical mastitis. Our hypothesis is that cows affected with severe infectious disease will have altered CEF PK relative to healthy cows, necessitating variance in dose regimens and withdrawal periods.

Materials and Methods

Animals and eligibility criteria

This study was completed at the Iowa State University (ISU) Dairy Farm. The ISU lactating herd consists of approximately 400 animals (approximately 90% Holstein and 10% Jersey), with 365-day rolling herd averages of 11,324 kg milk, 415 kg fat, and 357 kg protein. Throughout the trial, cows were housed in a free-stall barn bedded with recycled manure solids, which is standard practice for this dairy. Cows were fed a total mixed ration and watered *ad libitum*. Cows were milked three times daily at 4 am, 12 pm, and 8 pm. Cow housing and management met or exceeded the recommendations listed in the *Guide for Care and Use of Agricultural Animals in Research and Teaching* (FASS, 2010). The herd was vaccinated with a J5 core antigen vaccine 42 and 28 days prior to calving and again 25 and 90 days following calving. ISU's Institutional Animal Use and Care Committee approved the research protocol prior to commencement of trial procedures (protocol number 6-14-7820-B).

Eight cows that presented with naturally occurring, acute severe toxic clinical mastitis, as described by Wenz et al. (2001b), were selected to participate in the trial (disease group). The disease group had a mean (\pm SD) weight of 607 ± 77 kg (range 453-689 kg). The farm's milking crew first identified each disease group cow based on an acute drop in milk production, abnormal milk, and severe swelling in the affected gland. At enrollment, all cows had evidence of systemic clinical signs associated with endotoxemia. In order to qualify, cows had to present with: 1) at least one hot, swollen quarter secreting abnormal milk; 2) an acute decrease in milk production; and 3) evidence of systemic disease involvement determined by the presence of an elevated rectal temperature,

depression, dehydration, anorexia, and decreased blood circulation as determined by the presence of congested mucous membranes or delayed capillary refill time.

Upon identification of a disease cow with severe mastitis, a healthy herd mate was matched by breed, days in milk, and lactation number to serve as a control. The control group's mean (\pm SD) weight was 650 ± 100 kg (range 462-751 kg). Disease and control cows were eligible for the trial if they had not been treated with systemic or IMM CEF within the past 20 days and were healthy prior to enrollment. Furthermore, the cows needed to be ten or more days from their next scheduled dry period.

Study design

Disease and control cows were enrolled immediately following the noon milking. Throughout the trial, cows were milked by trial personnel per the farm's milking protocol. Prior to the noon milking of the disease group cows, a milk sample was aseptically collected from the mastitic quarter for bacterial culture. This sample was kept on ice until it was transferred to the laboratory for microbiological analysis.

After milking, disease and control cows were weighed and placed in treatment chutes; two 10-mL blood samples were collected from the jugular vein in blood tubes containing freeze-dried heparin for plasma harvest (Becton, Dickinson and Co., Franklin Lakes, NJ). Disease and control cows then received 2.2 mg CEF equivalents (CE) per kg of body weight in the form of CEF hydrochloride (Excenel RTU EZ, Zoetis, Florham Park, NJ) via intramuscular (IM) injection on the left side of the neck. Injections were given on alternating sides of the neck each day. In accordance with the manufacturer's recommendations, injection volumes were divided so that no more than 10 mL was

administered per injection site. CEF hydrochloride injections were repeated at approximately 24-hour intervals for five days. At the discretion of the treating veterinarian, ancillary therapies of flunixin meglumine (FLU) (2.2 mg/kg intravenously [IV]) (Prevail, VETONE, Boise, ID), 7.2% hypertonic saline (3-5 mL/kg IV) (Hypertonic Saline 7.2%, VETONE, Boise, ID), and oral fluids (20-40 L) were also provided for the disease group per standard treatment protocols in place for the dairy farm, as needed. The oral fluids consisted of tap water with 0.5 kg of an oral electrolyte powder (Fresh Cow Drench, IBA Inc., Millbury, MA) and 0.5 kg of dehydrated alfalfa meal added. The control cows received no ancillary therapy.

According to the farm's treatment protocols, intramammary (IMM) therapy was based on the results of bacterial culture. For cows with gram-negative mastitis, negative culture results, or agents determined to be non-responsive to IMM therapy (e.g., *Prototheca zopfii* or yeast), no IMM therapy was to be utilized. If a gram-positive agent was identified, IMM tubes that did not contain CEF were to be utilized according to label directions.

Blood samples were collected using venipuncture from the jugular vein into heparinized tubes at 0.33, 0.67, 1, 1.5, 2, 3, 4, 8, 16, and 24 hours after the first dose of CEF and approximately every 8 hours thereafter, concluding 120 hours after the last dose. After blood was collected, samples were immediately placed on ice until plasma could be harvested. Within one hour of collection, blood samples were centrifuged for ten minutes at 1000× g, and 5 mL of plasma was collected and frozen at -70°C until it was analyzed for CEF plasma concentration.

Milk samples were cultured on Brain Heart Infusion Agar containing 5% sheep blood and MacConkey Agar (Remel Microbiology Products, Lenexa, KS) using sterile,

cotton-tipped swabs. Plates were inverted, incubated at 37°C, and evaluated after 24 and 48 hours for bacterial growth. Bacteria were identified based on visual appraisal of the plates utilizing typical colony characteristics as described by the National Mastitis Council (1999).

Plasma ceftiofur concentration analysis

Plasma concentrations of CEF were determined using liquid chromatography coupled with mass spectrometry (LC-MS). The LC-MS system consisted of an Agilent 1100 pump, autosampler, and column compartment (Agilent Technologies, Santa Clara, CA) coupled to an ion trap mass spectrometer (LTQ, Thermo Scientific, San Jose, CA). Total CEF concentrations (expressed as CE) were determined by cleavage of CEF, its metabolites, and protein-bound residues to desfuroylceftiofur (DFC) using dithioerythritol (DTE), followed by derivatization with iodoacetamide. The resulting stable derivative, desfuroylceftiofur acetamide (DCA), was then analyzed by LC-MS. The internal standard was deuterated CEF (d3-CEF), which became d3-DCA upon cleavage and derivatization.

Clean up of the derivatized samples was performed by solid phase extraction (SPE) using Oasis HLB cartridges (Waters Corp., Milford, MA). Plasma samples, plasma calibrators, and quality control samples (200 µL) were treated with 3 mL of 0.5% DTE in borate buffer (0.05 N, pH 9.0) after addition of 10 µL of a 10 ng/µL solution of the internal standard, d3-CEF. The samples were then vortexed for 5 seconds and placed in a 50°C water bath for 15 minutes. Upon removal from the water bath and cooling to room temperature, 0.5 mL of 14% iodoacetamide in phosphate buffer (0.025 M, pH 7) was added, and the samples were left in the dark for 30 minutes.

Following derivatization, the samples were cleaned on an Oasis HLB SPE column (60 mg/3 mL) that was preconditioned with 1 mL of methanol followed by 1 mL of water. The sample was then transferred to the SPE column and allowed to pass slowly through the HLB column. The column was washed with a 1 mL portion of 5% (v/v) solution of methanol in water and then dried for 5 minutes with a flow of nitrogen. Elution of the derivatized samples was then performed with two 0.75-mL portions of 5% (v/v) acetic acid in acetonitrile. The eluate was dried at 50°C with a stream of nitrogen in a Turbopap evaporator (Biotage, Charlotte, NC).

The dry residue was then reconstituted with 100 µL of 25% (v/v) acetonitrile in water and vortexed, then diluted with 50 µL of water and vortexed again. The tube contents were transferred to an autosampler vial fitted with a glass insert and the injection volume was set to 15 µL. The mobile phases consisted of A) 0.1% formic acid in water and B) 0.1% formic acid in acetonitrile at a flow rate of 0.25 mL/min. The mobile phase began at 10% B with a linear gradient to 95% B at 8 minutes, which was maintained for 1.5 minutes, followed by re-equilibration to 10% B. Separation was achieved with an ACE C18 column (ACE 3 C18, 150 mm × 2.1 mm, 3 µm particles, Mac-Mod Analytical, Chadds Ford, PA, USA) maintained at 40°C. DCA was eluted at 3.70 minutes and the internal standard, d3-DCA, was eluted at 3.66 minutes.

Sequences consisting of plasma blanks, calibration spikes, quality control samples, and bovine plasma samples were then batch-processed with an automated processing method developed in the Xcalibur software (Thermo Scientific, San Jose, CA, USA), which identified and integrated each sample peak. The calibration curve was calculated based on a weighted (1/X), linear fit. Plasma concentrations of CEF in trial samples were

calculated based on this calibration curve. Results were then viewed in the Quan Browser portion of the Xcalibur software. Calibration spikes were utilized from 1 to 10,000 ng/mL. The standard curve had a linear range from 1 to 2000 ng/mL with a correlation coefficient of 0.998. All standards were within +/- 10 % of the nominal value in this range. A quadratic fit was needed to fit in the last two calibrators at 5000 and 10,000 ng/mL. The limit of detection was 0.4 ng/mL and the limit of quantification was 2 ng/mL.

Pharmacokinetic analysis

Due to the trial design, the PK data could not be modeled using only one model. Therefore, both a single dose and multi-dose PK analysis was performed with computer software using non-compartmental methods (Phoenix WinNonlin, Certara, L.P., Princeton, NJ, USA). For the single dose PK analysis, the maximum plasma concentration for the first dose ($C_{max_{1st\ dose}}$), time to C_{max} ($T_{max_{1st\ dose}}$), and area under the curve from 0-24 hours (AUC_{0-24}) among all cows was determined directly from plasma samples collected after the first dose of CEF until the second dose was administered. The terminal half-life of the first dose ($T_{1/2_{1st\ dose}}$) was determined from at least three time points on the terminal portion of the plasma profile before the second dose was administered.

To complete the multi-dose model, time points that were equally distributed were used, starting with $T=8$ hours and continuing with the time points every eight hours through the end of the trial. The maximum plasma concentration at steady state per dose administered (C_{max_D}) was determined by dividing steady state maximum plasma concentration ($C_{max_{ss}}$) by the actual dose administered. The terminal half-life following the last dose ($T_{1/2_{last\ dose}}$) was determined from at least six time points on the terminal portion

of the plasma profile after the last dose. The area under the curve at steady state for the dosing interval (AUC_{τ}) was determined directly from the data at 96–120 hours after the first dose of CEF. The volume of distribution (area method) per fraction of the dose absorbed (V_z/F) was calculated with the following equation:

$$\frac{Dose}{\lambda_z * AUC_{\tau}}$$

where λ_z represents the individual estimate of the terminal elimination rate constant, which is calculated by determining the log-linear regression of the terminal portions of the plasma concentration versus the time curves. The plasma clearance per fraction of the dose absorbed at steady state (CL/F) was calculated with the following equation:

$$\frac{Dose}{AUC_{\tau}}$$

The accumulation ratio was determined by dividing AUC_{τ} by AUC extrapolated to infinity after the last dose. The mean residence time (MRT) was calculated by dividing the area under the moment curve (AUMC) extrapolated to infinity after the last dose by the AUC extrapolated to infinity after the last dose.

Data analysis

Statistical analysis was performed using SAS 9.4 (SAS Institute, Cary, NC). All PK data are reported as geometric means. Disease and control groups were compared using the Wilcoxon two-sample rank-sum test (Mann-Whitney test). Multiple variables (i.e. drug concentrations and PK values) had distributions that were right skewed with long tails. Therefore, non-parametric methods were used to analyze the data in this situation. Statistical significance was established when $P < 0.05$.

Results

Eight cows (seven Holstein, one Jersey) were enrolled in the trial as disease group animals. At enrollment, disease group cows ranged from 2–5 lactations and 2–354 days in milk. All cultures from mastitis samples were pure cultures and identified as either *E. coli* (n=5) or *Klebsiella* spp. (n=3). Following the 10-day trial period, five of the eight disease group cows were immediately culled from the herd and one additional cow was culled within 60 days; all culls resulted from the clinical mastitis episode that initiated their enrollment in the trial. The remaining two cows, one with mastitis caused by *E. coli* and one by *Klebsiella* spp., remained in the herd for at least 60 days following the clinical episode of mastitis. There were no statistical differences in any comparative parameters between disease and control cows (Table 1).

Seven of the eight disease group cows received ancillary therapy of FLU for an average of 2.3 days (range 1-5), five of the eight received hypertonic saline an average of 1.2 days (range 1-2), and all eight received oral fluids an average of 3.9 days (range 2-9). None of the cows received IMM antimicrobial therapy.

No cow had detectable plasma CEF at enrollment (time 0) and CEF was detected in all subsequent samples throughout the entire study period. CEF concentrations were significantly higher in the disease group 3 hours after the first injection and then significantly lower at 40, 48, 56, 72, 80, 88, 96, 104, 112, 120, 128, 136, 144, and 152 hours after the first injection (Figures 1 and 2).

Several PK parameters differed between disease and control cows. The results of the single dose PK model are displayed in Table 2 and those from the multi-dose model

are displayed in Table 3. Compared to control cows, the single dose PK model indicated a shorter $T_{1/2\text{1st dose}}$ ($P = 0.038$) for the disease group. The multi-dose PK model indicated that the disease group had a higher V_z/F ($P = 0.001$) and CL/F ($P = 0.0006$) compared to the control group. The disease group had lower AUC_{tau} ($P = 0.0006$), $C_{\text{max}_{\text{ss}}}$ ($P = 0.0006$), and C_{max_D} ($P = 0.0006$). There was also a tendency for a longer $T_{1/2\text{ last dose}}$ in the disease group ($P = 0.065$). All other PK parameters were not different between the two groups in both models.

Discussion

Ceftiofur is a third-generation cephalosporin marketed for use in multiple food animal species. In the dairy industry, CEF is marketed for systemic and IMM use. Based on our experience and that of others (Zwald et al., 2004; Sawant et al., 2005), CEF is likely the most commonly used antimicrobial in the United States dairy industry due to its broad spectrum of activity, multiple formulations, and short milk and meat withholding periods. Within an hour of injection with CEF products, the parent compound is almost completely metabolized by the liver to DFC and small concentrations of other non-polar and polar metabolites (Jaglan et al., 1989; S. A. Brown et al., 1991). DFC has an exposed sulfhydryl group that forms reversible disulfide bonds with other sulfurous compounds, such as sulfhydryl-containing proteins and glutathione. These protein-bound CEF metabolites are not biologically active but can dissociate quickly in reduced environments to form active metabolites (Clarke et al., 1996). In cattle, protein binding is age-dependent, with low levels in calves while healthy adult cows have CEF metabolites that are >90% bound (S. A. Brown et al., 1991). Repeated dosing with CEF may result in saturation of protein

binding sites, resulting in the presence of more unbound, active metabolites over time without increasing $T_{1/2}$ (Halstead et al., 1992). More than 95% of administered CEF sodium is excreted within 24 hours; with 61-77% of the total drug excreted through urine and the balance excreted in feces (S. A. Brown et al., 1991). Drug approval studies show that metabolism and excretion of CEF hydrochloride by cattle is similar to that of CEF sodium (US FDA, 1998), and tissue distribution of CEF is similar regardless of the CEF salt administered (US FDA, 1998; KuKanich et al., 2005).

Over the last several years, concerns about veterinary drug use, its impact on antimicrobial efficacy in humans, and the presence of drug residues in milk and meat have increased. This is especially true for medically important drugs, including cephalosporins (US FDA, 2006b). In 2012, the FDA issued a prohibition against extra-label drug use of cephalosporins in bovines for those cephalosporins with analogs in human medicine (US FDA, 2012b). While mastitis is an extra-label condition for all systemic CEF products, there are no FDA restrictions on extra-label conditions as long as the dose, duration, and route of administration label conditions are followed (US FDA, 2012b). Using published literature, (e.g. Wenz et al. (2001a) and Erskine et al. (2002)) veterinarians can justify this extra-label therapy provided it is in compliance with the requirements of the Animal Drug Use Clarification Act (US FDA, 1996).

In the dairy industry, CEF is primarily affected by the FDA prohibition; however, CEF residues have increased substantially since FY2011. Between FY2011 and 2012, confirmed violative residues from inspector-generated samples in culled dairy cattle increased from 53 to 130 (USDA 2013, 2014a), rising to 283 in FY2014 (USDA, 2014b). These reports indicate that CEF residues are now the most common violative residues

found in cull dairy cattle. Given the extra-label prohibition and the importance of the drug to the dairy industry, there is extreme concern about this level of violative residues. Although the reason for this level of violative residues is likely multi-factorial, one potential cause may be altered PK and residue depletion of CEF in diseased dairy cattle.

The objective of this trial was to determine if plasma PK for CEF were different between healthy cows and cows with severe disease. The findings suggest that disease may indeed alter the PKs of CEF in diseased cattle, resulting in a shorter $T_{1/2\text{ 1st dose}}$ in the disease group when the data was modeled for a single dose. When multi-dose modeling was applied, there was more than a two-fold increase in V_z/F and a nearly two-fold increase in the plasma clearance rate. Additionally, $C_{\text{max ss}}$, $C_{\text{max D}}$, and AUC were significantly lower in the disease group, resulting in more than a 40% reduction in each parameter. Following the first injection, our single PK data was close for $C_{\text{max 1st dose}}$ and $T_{\text{max 1st dose}}$ compared to package insert data supplied with the product (Zoetis, 2013a). However, the $T_{1/2\text{ 1st dose}}$ data was substantially shorter than the package insert (8.13 hours for the control group vs. 29.3 hours from the package insert). The likely explanation for this difference was the limited amount of time available ($T=3$ hours to $T=24$ hours) to follow the depletion curve after the $C_{\text{max 1st dose}}$ before the second dose was administered. During that time, the depletion curve was fairly linear unlike the depletion curve that occurred following the fifth dose, leading to a much shorter depletion estimate for the single PK model (see Figure 1). In contrast, the $T_{1/2\text{ last dose}}$ was much closer to the value reported in the package insert.

A weakness of the multi-dose PK model was that the first time point ($T=8$ hours) to be included in this model occurred after the $C_{\text{max 1st dose}}$ and $T_{\text{max 1st dose}}$. This biased the reported value for AUC_{tau} lower. As a result, the values for V_z/F and CL/F are over-

estimates in the data reported. However, since this occurred in both groups and $C_{max_{1st\ dose}}$ and $T_{max_{1stdose}}$ were not statistically different, the changes should be proportional between the two groups, indicating that while the values are not exact, the change between the two groups for AUC_{tau} , V_z/F , and CL/F are true. This can be verified by studying the concentration curves presented in Figure 2 and noting that a statistically significant change in concentrations started to occur at $T=40$ hours through $T=152$ hours, with the exception of $T=64$ hours.

Many physiological changes could explain the PK changes we found, including altered absorption of the drug due to altered blood flow to the injection site; reduced plasma protein levels in diseased animals that allowed unbound CEF to move more freely to tissue or be excreted; competition with co-administered drugs (e.g., FLU) for protein binding sites; changes in membrane permeability in tissue, such as the mammary gland; altered liver and kidney function due to altered blood flow or altered enzyme systems (van Miert, 1990); or altered function of membrane-bound drug transporters (Petrovic et al., 2007).

Despite the widespread use of CEF in dairy cattle, there is a paucity of data comparing PK parameters of CEF between healthy and diseased cows. In one trial, Erskine et al. (1995) compared drug concentrations in healthy cows versus cows experimentally challenged with IMM *E. coli*; however, they utilized a different dosing strategy (3 mg/kg IV every 12 hours for 3 treatments), which resulted in all drug administration occurring within 24 hours. In accordance with our findings in the single dose PK model, they reported no difference in peak concentrations of CEF in the serum of treated vs. healthy control cows. In their trial, they found higher serum concentrations of active CEF metabolites in the control group, suggesting that diseased cattle had increased distribution out of the

plasma pool. We found a similar increase in plasma distribution of drug, but we did not measure active compound. However, in contrast to our results, they reported peak serum concentrations 90% less than the values measured in our trial. This disparity may be due to methodological differences; their assay did not account for protein-bound metabolites, which could make CEF concentrations appear low.

Other researchers have compared CEF concentrations using both high performance LC and microbiological assay in feedlot cattle implanted with tissue chambers; half of the chambers were later inoculated with *Mannheimia haemolytica*. Both methodologies indicate that total DCA is significantly higher in infected than uninfected tissue chambers, even within a single animal. Interestingly, the ratio of active CEF to total DCA was higher in the infected chamber than uninfected chamber (Clarke et al., 1996). Thus, it appears that a higher concentration of total CEF (both bound and active) accumulates at the site of infection by passively moving through disrupted endothelial cell barriers and from the binding of CEF to acute phase proteins, such as α_1 -anti-trypsin, which rapidly move to sites of infection (Clarke et al., 1996; Walker et al., 1994). There could also be impairment of outward fluxing membrane transporter pumps due to inflammation (Petrovic et al., 2007). Movement through impaired membranes of the mammary gland may explain the higher V_z/F in diseased cattle described in our study.

Other comparisons of antimicrobial PK in diseased versus healthy animals have been completed in swine (Tantituvanont et al., 2009; Day et al., 2015) and chickens (Amer et al., 1998). The swine trials included pigs experimentally infected with porcine reproductive and respiratory syndrome virus (PRRSV) (Tantituvanont et al., 2009) and pigs co-infected with PRRSV and *Streptococcus suis* (Day et al., 2015), both of which were

compared to healthy control animals. In these trials, volume of distribution and CL were higher and plasma concentration values lower in diseased than healthy animals (Tantituvanont et al., 2009; Day et al., 2015). In the poultry trial, one group of healthy chickens was exposed to aflatoxin in their diet while the control group was fed a diet devoid of aflatoxin. Both groups were treated with CEF via the IV, IM or oral route. In the chickens treated via the oral and IV route, tissue concentrations of CEF were lower and CEF was eliminated more quickly (Amer et al., 1998). Taken together, these three trials (Tantituvanont et al., 2009; Day et al., 2015; Amer et al., 1998), combined with our work, suggest that more research on drug disposition is needed in disease-challenged animals. Future trials for drug approval should investigate the effect of disease when establishing therapeutic dosages and residue depletion time.

In this trial, the goal was to approximate treatment strategies utilized on commercial dairy farms. Therefore, research personnel did not interfere with treatment decisions regarding co-administered therapies. Administration of CEF to cows with moderate or severe mastitis reduces culling as a result of the mastitis event (Erskine et al., 2002). Therefore, it is included in treatment protocols for mastitis by many veterinarians. FLU is commonly included in the treatment of moderate to severe clinical mastitis (Kissell et al., 2015), despite inconsistent evidence of clinical benefits (Dascanio et al., 1995; Anderson et al., 1986; Wagner and Apley, 2004; Yeiser et al., 2012). Admittedly, the co-administration of CEF and FLU potentially confounds the outcome. FLU is reported to be greater than 98% protein bound (Anderson, 1990; Odensvik, 1995) which may actively compete with CEF for the same protein binding sites. While the PK changes that occur with the co-administration of CEF and FLU are not known, theoretically protein binding

could be reduced for one or both drugs. If CEF was less protein bound as a result of co-administration of FLU, this could account for the changes that were noted for V_z/F and CL. However, in a pair of manuscripts, Whittem et al. (1995 and 1996) reported no PK differences with ceftiofur or aspirin following co-administration of single doses of each drug. Like FLU, aspirin is a non-steroidal anti-inflammatory drug that achieves high protein binding in the bovine (Lees, 2009). Additional research is needed to determine the effects of highly protein-bound drugs co-administered with other such drugs, including the co-administration of CEF and FLU, because this is how the drugs are typically used on dairy farms.

In conclusion, these data, coupled with that of other researchers, show that significant PK changes occur in diseased animals administered CEF relative to healthy, control animals. These outcomes potentially have public health significance in that: 1) drug efficacy could be lower than expected; 2) there may be an increase in violative drug residues in tissues of cull animals or milk; and 3) this may lead to increases in antimicrobial resistance. This body of evidence suggests that the drug approval process should be changed such that the physiological changes of health-challenged cows are addressed. It does not, however, lead to any conclusions regarding the outcomes of treating health-challenged cows nor the contribution of altered PK on the increasing violative CEF residues found in culled dairy cattle. Further work examining protein binding, tissue distribution, and tissue depletion of drugs and their influence on residue levels in diseased animals at the end of their withdrawal periods is necessary to more thoroughly characterize this problem.

Table 1. Mean \pm SD days in milk, lactation number, cow weight, and milk production for eight severe mastitis cows (disease group) and eight control cows.

Parameter	Disease group	Control group
Days in milk	119.2 \pm 122.6	119.5 \pm 125.6
Lactation number	3.4 \pm 0.9	3.1 \pm 1.0
Age of cow (yr)	5.0 \pm 1.4	4.6 \pm 1.3
Cow weight (kg)	607 \pm 77	650 \pm 100
305-day mature equivalent milk production (kg)	10246 \pm 1118	9996 \pm 1352

Table 2. Single dose plasma pharmacokinetic parameters of ceftiofur equivalents after the first dose for disease and control cows using a multi-dose regimen. Results are presented as geometric mean for ceftiofur equivalents¹.

Parameter	Disease group ²	Control group
Cmax _{1st dose} (μ g/mL)	11.75 (7.73-17.80)	9.58 (7.73-13.26)
Tmax _{1st dose} (h)	2.54 (2-4)	2.87 (2-4)
T1/2 _{1st dose} (h)	6.57 (5.04-8.04)	8.13 (6.59-9.80)*
AUC ₀₋₂₄ (h \times μ g/mL)	106.4 (85.94-124.54)	95.43 (63.82-117.00)

¹Values in parentheses are the range.

²Cows with severe toxic mastitis.

Cmax = maximum plasma concentration for the first dose; Tmax = time to Cmax; T1/2_{1st dose} = terminal half-life of the first dose; AUC₀₋₂₄ = area under the concentration curve from 0-24 hours.

*Geometric means within the columns differ ($P < 0.05$).

Table 3. Multi-dose plasma pharmacokinetic parameters of disease and control cows. Results are presented as geometric mean for ceftiofur equivalents¹.

Parameter	Disease group ²	Control group
V _z /F (L/kg)	3.08 (1.96-5.18)	1.44 (1.08-2.19)**
CL/F (mL/min/kg)	0.850 (0.61-1.06)	0.466 (0.334-0.666)**
MRT (h)	23.6 (20.5-30.0)	22.3 (18.4-27.4)
AUC _{tau} (h × µg/mL)	44.7 (36.0-63.0)	81.2 (56.6-114.4)**
Cmax _{ss} (µg/mL)	3.28 (2.87-4.26)	5.56 (3.44-7.31)**
Cmax _D (µg/mL)	1.44 (1.25-1.84)	2.45 (1.52-3.19)**
T _{1/2 last dose} (h)	41.9 (33.7-70.9)	35.8 (31.2-40.5)
Accumulation ratio	1.55 (1.43-1.80)	1.48 (1.33-1.67)

¹Values in parentheses are the range.

²Cows with severe toxic mastitis.

V_z/F = volume of distribution per fraction of the dose absorbed; CL/F = plasma clearance per fraction of the dose absorbed at steady state; MRT = mean residence time; AUC_{tau} = area under the curve at steady state for the dosing interval; Cmax_{ss} = steady state maximum plasma concentration; Cmax_D = maximum plasma concentration at steady state per dose administered; T_{1/2 last dose} = terminal half-life following the last dose.

**Geometric means within the columns differ ($P < 0.005$).

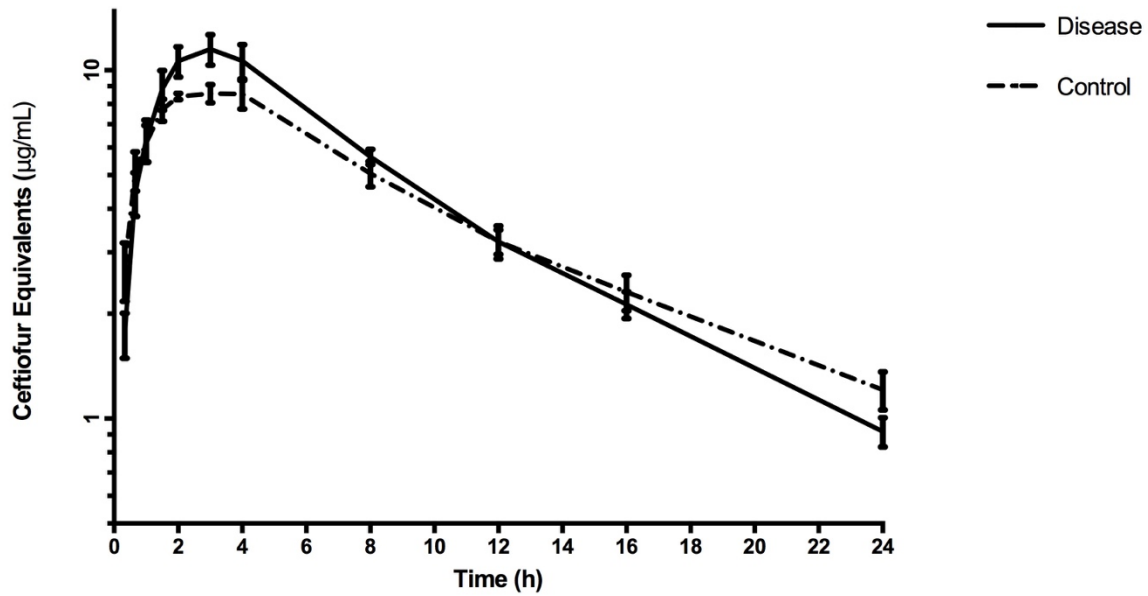


Figure 1. Log transformations of mean plasma ceftiofur equivalents (CE) concentration (± 1 SE) for eight disease group cows with severe mastitis and eight healthy control cows following a single administration of ceftiofur hydrochloride at 2.2 mg CE/kg of body weight.

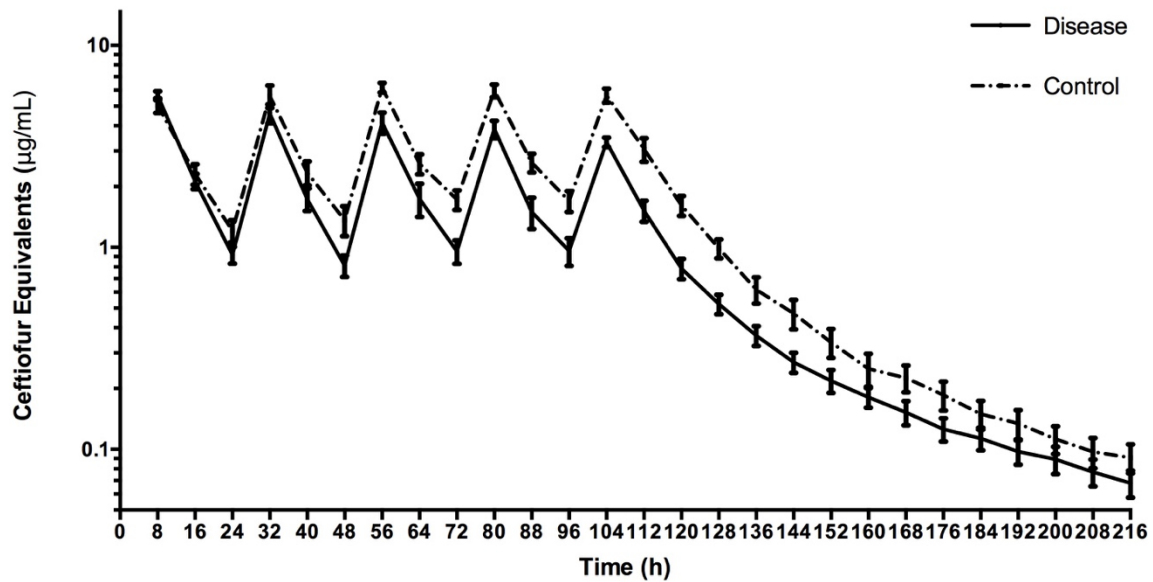


Figure 2. Log transformations of mean plasma ceftiofur equivalents (CE) concentration (± 1 SE) for eight disease group cows with severe mastitis and eight healthy control cows following five administrations of ceftiofur hydrochloride at 2.2 mg CE/kg of body weight.

CHAPTER 3.

COMPARATIVE PLASMA AND INTERSTITIAL FLUID

PHARMACOKINETICS OF FLUNIXIN MEGLUMINE AND CEFTIOFUR

HYDROCHLORIDE FOLLOWING INDIVIDUAL AND CO-ADMINISTRATION

IN DAIRY COWS

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Abstract

Ceftiofur (CEF) and flunixin meglumine (FLU) are two drugs approved for use in beef and dairy cattle that are frequently used in combination for many diseases. These two drugs are the most commonly used in dairy cattle in their respective drug classes. Two research groups have recently published manuscripts demonstrating altered pharmacokinetics of FLU and CEF in cows affected with naturally occurring mastitis. The objective of this study was to determine if pharmacokinetics of flunixin meglumine administered intravenously or intramuscularly administered ceftiofur hydrochloride would be altered when co-administered versus individual administration to healthy dairy cattle. Ten cows were utilized in a three-period, three-treatment crossover design, with all cows receiving each treatment one time with a 10-day washout period between treatments. Following treatment, plasma and interstitial fluid samples were collected and stored for later analysis. Additionally, plasma ultrafiltrate was collected using microcentrifugation to determine plasma protein binding of each drug. Drug concentrations in plasma, plasma ultrafiltrate, and interstitial fluid were determined using high pressure liquid chromatography coupled with mass spectrometry. The results of this trial indicate that drug interactions between FLU and CEF do not occur when the two drugs are administered simultaneously in healthy cattle. Further work is needed to determine if this relationship is maintained in the presence of severe disease.

Key words: flunixin meglumine, ceftiofur, pharmacokinetics, drug residues, dairy cattle

Introduction

Ceftiofur (CEF) and flunixin meglumine (FLU) are two drugs approved for use in beef and dairy cattle that are frequently used in combination for many diseases, including mastitis, metritis, and pneumonia (Schuler et al., 2017). Ceftiofur is a third-generation cephalosporin antimicrobial that is the most widely used antimicrobial in the dairy industry (Swant et al., 2005; Zwald et al., 2004; Schuler et al., 2017), due to its broad spectrum of antimicrobial activity and short withdrawal periods for milk and meat. Flunixin meglumine is a non-steroidal anti-inflammatory drug (NSAID) approved for use in lactating dairy cattle with indications for control of pyrexia associated with bovine respiratory disease, endotoxemia, and acute bovine mastitis. Additionally, FLU is labeled to control inflammation associated with endotoxemia. Flunixin meglumine is the most commonly prescribed analgesic in cattle in the US (Fajt et al., 2011).

During the drug approval process, sponsoring companies must present the FDA Center for Veterinary Medicine (CVM) with toxicological and residue depletion studies. Based on these data, the FDA CVM establishes withdrawal periods for meat and milk, if approved for lactating dairy cattle. However, these studies are performed on healthy animals, not animals suffering from infectious diseases. Additionally, there is only one drug administered at a time in these trials (US FDA, 2006b). During veterinary treatment of severe clinical disease (e.g., mastitis or metritis) in the lactating dairy cow, multiple drugs, like CEF and FLU, are often administered simultaneously to combat the clinical signs of the disease in question (Schuler et al., 2017). As most veterinary drugs are not intended for use in healthy animals, data on drug metabolism in diseased animals would provide veterinarians with evidence to more accurately prescribe veterinary drugs and to

better predict residue depletion in these diseased animals. Furthermore, data examining drug metabolism in sick animals and how this compares with healthy animals are deficient in the veterinary literature. Recently, two research groups have published manuscripts demonstrating altered pharmacokinetics (PK) of FLU (Kissell et al., 2015) and CEF (Gorden et al., 2016) in cows affected with naturally occurring mastitis. In both trials, mastitic cows received both FLU and systemic CEF simultaneously. In plasma, FLU (Anderson et al., 1990; Odensvik et al., 1995) and metabolites of CEF (S. A. Brown et al., 1991) are both reported to be >90% protein bound in adult cattle, leading to a question as to whether co-administration of two highly protein bound drugs may result in an interaction affecting protein binding of one or both drugs.

The objective of this study was to compare the plasma and interstitial fluid (ISF) concentrations; plasma protein binding; and plasma PK of CEF and FLU in healthy dairy cattle when administered separately versus concurrently. Our hypothesis was that co-administration of CEF via the intramuscular route and FLU via the intravenous route would result in altered plasma and ISF concentrations and altered PK relative to individual administration, necessitating variance in dose regimens or withdrawal periods.

Materials and Methods

Experimental cattle

This study was completed at the Iowa State University Dairy Farm. The lactating herd consists of approximately 400 animals (approximately 90% Holstein and 10% Jersey), with 365-day rolling herd averages per cow of 11,026 kg milk, 399 kg fat, and 341 kg protein. Ten Holstein cows were utilized in a three-period, three-treatment crossover design

with all cows receiving each treatment one time with a 10-day washout period between treatments. Cows were eligible for the trial if they had not been treated with systemic FLU and systemic or IMM CEF within the past 20 days and were healthy prior to enrollment. Furthermore, the cows were thirty or more days from their next scheduled dry period. The cows were in their 2nd lactation with a mean (\pm SD) days in milk of 181.7 ± 19.7 (range 136-207 days in milk) with a mean (\pm SD) daily milk production of 39.1 ± 5.7 kg (range 27.6-47.1 kg). The cows had a mean (\pm SD) weight of 654.2 ± 17.5 kg (range 573.3-722.2 kg).

During each treatment period, cows were housed in individual box stalls bedded with deep, long-stem straw. Each stall had individual access to feed and water. Cows were milked three times daily (4 am, 12 pm, and 8 pm). During the treatment periods, trial personnel milked trial cows per the farm's milking protocol. Between treatments, cows were housed in a free-stall barn bedded with recycled manure solids, which is standard practice for this dairy. Cows were fed a total mixed ration and watered *ad libitum*. Ration parameters met or exceeded those recommended by the NRC guidelines (NRC, 2001). Cow housing and management met or exceeded the recommendations listed in the *Guide for Care and Use of Agricultural Animals in Research and Teaching* (FASS, 2010). Iowa State University's Institutional Animal Use and Care Committee approved the research protocol prior to commencement of trial procedures (protocol number 6-14-7820-B).

Experimental design

Cows received each of the three treatments with a ten-day washout period between the treatments. Prior to the first replicate, the cows were randomly assigned to one of three

treatment groups using the RAND function in a spreadsheet program. Cows remained in the same groups for all three replicates. Assignment of the treatment at each replicate was also randomly assigned. The treatments were: FM = one dose flunixin meglumine (FLU) at 2.2 mg/kg IV (Prevail, VETONE, Boise, ID); C = one dose CEF (Excenel RTU EZ, Zoetis, Florham Park, NJ) at 2.2 mg ceftiofur equivalents (CE)/kg IM; and CF = one dose FLU at 2.2 mg/kg IV and one dose CEF at 2.2 mg CE/kg IM administered simultaneously. In accordance with the manufacturer's recommendations, CEF injection volumes were divided so that no more than 10 mL was administered per injection site.

One day prior to each treatment, cows were weighed and moved to their box stall. Intravenous catheters were placed in one jugular vein of all cows to facilitate blood collection. Cows that were to receive FLU during the replicate received a second catheter in the opposite jugular vein. Following restraint in a stanchion, cows were sedated with xylazine at approximately 0.025 mg/kg IV; the skin over the jugular furrow was clipped and aseptically prepped using alternating scrubs of 2% chlorhexidine acetate and 70% isopropyl alcohol. Prior to catheter placement, the area under the skin was infiltrated with 2% lidocaine. Following catheter placement, the catheter was sutured in place using #3 nylon suture. To maintain catheter patency, 3 mL of a heparin saline solution containing 3 USP units of heparin sodium/mL was infused into the catheters every 8 hours until treatments were initiated.

While sedated, all cows had one subcutaneous, in vivo ultrafiltration probe (RUF 3-12, BASi, West Lafayette, IN) placed dorsal-caudal to the scapula to facilitate ISF collection. Briefly, the area was prepped as described above and the probe was placed by passing a 10-gauge introducer needle between two small stab incisions through the skin.

Following probe placement, the collection tube was stitched in place and connected to a 7-mL red top glass vacuum tube (Becton, Dickinson and Co, Franklin Lakes, NJ) for ISF collection.

Collection of blood and interstitial fluid samples

At T0 hour, two 10-mL blood samples were collected from the jugular catheter into blood tubes containing freeze-dried heparin (Becton, Dickinson and Co, Franklin Lakes, NJ) for plasma harvest. Subsequent blood samples were collected from the jugular catheter into heparinized tubes at 0.083, 0.166, 0.33, 0.5, 0.75, 1, 1.5, 2, 4, 6, 8, 12, 18, 24, 30, 36, 42, 48, 54, 60, 66, and 72 hours after drug administration. After blood was collected, samples were immediately placed on ice until plasma could be harvested. Within 2 hours of collection, blood samples were centrifuged for 20 minutes at $1000\times g$ at 4 °C, then 5 mL of plasma was harvested and frozen at -70 °C until analyzed for drug concentration.

Simultaneous with drug administration (time 0), a new vacuum tube was attached to the ultrafiltration probe. Interstitial fluid samples were collected at 1, 2, 4, 6, 8, 12, 18, 24, 30, 36, 42, 48, 54, 60, 66, and 72 hours following drug administration by changing the vacuum tube. The tubes were immediately frozen at -70 °C until analyzed for drug concentration.

Determination of plasma protein binding

Bound plasma drug concentration of FLU and CEF was determined on each cow on the T4 hour plasma samples using a microcentrifugation system (Centrifree Ultrafiltration Device, EMD Millipore Corp., Billerica, MA). The microcentrifugation

device had a molecular weight cut-off of 30,000. Following plasma harvest, 1 mL of plasma was transferred to the microcentrifugation device and then the device was centrifuged at 1700× g for 30 minutes. The collection cups were then capped and frozen at -70 °C until analyzed for drug concentration. These samples were analyzed for drug concentration in the same manner as for ISF samples described below, except spike and quality control (QC) samples were prepared using blank plasma. For FLU, the spike range was from 3 to 5000 ng/mL, with QC samples at 30, 300, and 3000 ng/mL. For CEF, the spike range was from 1 to 5000 ng/mL, with QC samples at 15, 150, and 1500 ng/mL. Following determination of drug concentration of both the plasma and the microcentrifugation sample containing unbound drug, protein-binding percentage was determined using the following formula:

$$\% \text{ protein binding} = \left[\frac{(\text{total drug} - \text{unbound drug})}{\text{total drug}} \right] * 100$$

Plasma and interstitial fluid ceftiofur concentration analysis

Plasma and ISF concentrations of CEF were determined using liquid chromatography coupled with mass spectrometry (LC-MS) as described in Gorden et al. (2016). The limit of detection (LOD) was 1 ng/mL, and the limit of quantification (LOQ) was 10 ng/mL. The accuracy and coefficient of variation for the quality control (QC) samples were 97% and 11.2% for the 15 ng/mL QC; 96% and 6.4% for the 150 ng/mL QC; and 95% and 9.3% for the 1,500 ng/mL QC.

Plasma and interstitial fluid flunixin concentration analysis

Plasma and ISF concentrations of FLU were determined using LC-MS as previously described (Kleinhenz et al., 2016). The LOD was 3 ng/mL and the LOQ was 5 ng/mL. The accuracy and coefficient of variation for the QC samples were 106% and 4.4% for the 30 ng/mL QC; 112% and 4.2% for the 300 ng/mL QC; and 111% and 4.2% for the 3,000 ng/mL QC.

Pharmacokinetic analysis

The plasma drug concentration-time profiles were analyzed using non-compartmental methods implemented in a commercially available software program (Phoenix WinNonLin 6.4, Certara, Cary, NC, USA). Pharmacokinetic parameters calculated for FLU with this method included: λ_z (1/h), slope of the terminal phase; $T_{1/2 \lambda_z}$ (h), terminal half-life; C_0 ($\mu\text{g/mL}$), the initial concentration immediately after drug administration calculated by back-extrapolating from the straight line model fit to the first two measured concentrations following IV administration; $\text{AUC}_{0-\text{tlast}}$ ($\text{h} \cdot \mu\text{g/mL}$), area under the curve from T0 to the last detectable concentration; $\text{AUC}_{0-\infty}$ ($\text{h} \cdot \mu\text{g/mL}$), area under the curve extrapolated to infinity using the equation $\frac{C_{\text{last}}}{\lambda_z}$; V_{area} (mL/kg), volume of distribution based on the terminal phase of the time-concentration curve; CL (mL/h/kg), clearance; and MRT (h), mean residence time. For CEF, λ_z ; $T_{1/2 \lambda_z}$; C_{max} ($\mu\text{g/mL}$), maximum plasma concentration; T_{max} (h), time of C_{max} ; $\text{AUC}_{0-\text{tlast}}$; $\text{AUC}_{0-\infty}$; V_{area}/F , volume of distribution per fraction of the dose absorbed; and CL/F , clearance per fraction

of the dose absorbed; and MRT were determined. Both AUC and MRT were extrapolated to infinity to account for the total exposure to the drug.

Data analysis

Statistical analysis was performed using a commercially available software program (JMP Pro 11.0.0, SAS Institute, Cary, NC). All data is expressed as arithmetic mean \pm SE and geometric mean. Comparison of plasma concentrations for each drug over time were evaluated using repeated measures in a multivariate analysis of variance analysis. Drug concentrations were compared at each time point using contrasts. Comparison of variables between treatment groups that were single observations (i.e., enrollment variables and PK parameters) were made using a paired t-test when data were normally distributed and with a Wilcoxon signed rank test when distributions were not normally distributed (FLU variables λ_z and $T_{1/2 \lambda_z}$). Comparisons of ISF concentrations at each time point were made using the Wilcoxon signed ranked test by time if there were sufficient sample to facilitate analysis. Statistical significance was established when $P < 0.05$.

Results

At enrollment, there was no statistical difference for any of the animal enrollment variables between the treatment groups.

No cow had detectable FLU or CEF in plasma or ISF at the beginning of each replicate (time 0). Plasma and ISF concentrations for FLU are displayed in Figure 1. Following drug administration, FLU was detected in all subsequent plasma samples

through 18 hours. No cow had quantifiable FLU concentrations in plasma past 30 hours. For the ISF samples, appearance of FLU concentrations above the LOQ was variable, with no cows having quantifiable values 1 hour after FLU administration and only 4 having quantifiable values at 2 hours after FLU administration. One cow had no quantifiable ISF FLU in both treatments (FM and CF) at any time point. At eleven time points throughout the trial, there was insufficient sample volume available to complete the analysis. No cow had quantifiable FLU concentrations in ISF past 30 hours. There were no significant differences in plasma or ISF concentrations between the FM and CF treatment groups throughout all the time points measured, except at the T2 hour time point for plasma FLU concentration (FM=0.52 $\mu\text{g/mL}$ vs. CF=0.33 $\mu\text{g/mL}$, $P = 0.005$). Pharmacokinetic parameters for FLU are displayed in Table 1. There were no significant differences for PK variables between the FM and CF treatments for FLU.

Plasma and ISF concentrations of CEF are displayed in Figure 2. Following drug administration, CEF was detected in all subsequent plasma samples throughout the entire study period. For the ISF samples, none of the cows had CEF concentrations above the LOQ at one hour after drug administration and only 3 cows had quantifiable CEF concentrations in their ISF by 2 hours post-administration. At four hours after therapy, all but one cow (in the CF treatment group) had quantifiable CEF concentrations in their ISF, with CEF depleted below the LOQ from the ISF of all cows by 72 hours after treatment. There were no significant differences in plasma or ISF CEF concentrations between the C and CF treatment groups throughout all the time points measured. Pharmacokinetic parameters for CEF are displayed in Table 2. There were no significant differences for PK variables between the C and CF treatments for CEF.

Mean protein binding (± 1 SE) for FLU at T4 h was $98.6\% \pm 0.19$ for the FM treatment and $98.6\% \pm 0.06$ for the CF treatment. Mean and protein binding (± 1 SE) for CEF at T4 hours was $94.6\% \pm 0.003$ for both treatments. There were no significant differences in protein binding between any of the treatments.

Discussion

This trial utilized only cattle from the Holstein breed and only in their second lactation. This was done to reduce any variation that may have been introduced by breed or age differences in drug metabolism.

Flunixin meglumine and CEF are two veterinary drugs that are often used in combination for the treatment of severe infectious processes, such as mastitis or metritis (Schuler et al., 2017). Kissell et al., (2015) recently demonstrated that cows treated with FLU and CEF for clinical mastitis had different metabolism for FLU than healthy cows, necessitating a recommendation for an increased milk withdrawal time. Our group recently undertook a similar trial and demonstrated that cows with severe clinical mastitis had higher volume of distribution and plasma clearance of CEF in comparison to healthy cows (Gorden, et al., 2016). One potential explanation for this phenomenon is that the co-administration of FLU and CEF may have resulted in an interaction that interfered with protein binding, as both drugs are better than 90% protein bound in plasma (Anderson et al., 1990; Odensvik et al., 1995; S. A. Brown et al., 1991). The objective of this trial was to compare the plasma and tissue concentrations and plasma PK of CEF and FLU in healthy dairy cattle when administered separately versus concurrently. Based on the results of this trial, the data do not support our hypothesis that drug metabolism would be affected by a

drug interaction between FLU and CEF, at least in healthy animals. This finding is consistent with that reported by Whitem et al. (1995 and 1996) in a pair of manuscripts showing no difference in PK of ceftiofur or aspirin, another NSAID, following co-administration of single doses of each drug.

Compared with the FLU PK parameters reported by Kissell et al. (2015), similar values were found for MRT but higher values were found for V_{area} and CL. This trial also reported substantially lower values for AUC. Kissell et al. (2015) reported a mean peak plasma concentration of 18.35 $\mu\text{g/mL}$, which occurred 15 minutes following injection and not being able to find flunixin in plasma after 12 hours following treatment. Our initial plasma observed concentration (C_0) was determined to be $22.62 \pm 1.06 \mu\text{g/mL}$ and $20.52 \pm 2.22 \mu\text{g/mL}$ for the FM and CF treatments, respectively. However, by 10 minutes after FLU injection, mean plasma values had already dropped to $10.84 \pm 0.97 \mu\text{g/mL}$ and $12.49 \pm 0.73 \mu\text{g/mL}$ for the FM and CF treatments, respectively. Flunixin was detected in plasma samples up to 30 hours post-treatment, 18 hours longer than reported by Kissell et al. (2015), however lower LOD and LOQ levels were implemented in this trial. Mean FLU protein binding was determined to be 98.6% in both treatment groups, which is slightly lower than values reported by Anderson, et al. (1990) and Odensvik, et al. (1995). This change, while small, may have a dramatic effect on PK of FLU. A change of 1% would approximately double the unbound FLU, making it more available to distribute out of the plasma and thus alter the volume of distribution and clearance.

Regarding the CEF treatments, mean C_{max} and T_{max} values were approximately equal to healthy cows from our previous work and those reported on the package insert supplied with the product. Additionally, the mean CL/F was similar to our previous data.

However, the $T_{1/2}$ determined here is much shorter than the previous work or the package insert. The current work also had a higher AUC and lower V_{area} and MRT than the previous work (Gorden et al., 2016; Zoetis, 2013a). These differences are potentially due to variation in animals used between our trials, differences in types of animals used in our trial and the manufacturer's trials, and differences in assay sensitivity between our trials and those of the manufacturer. Protein binding of CEF in both treatments was determined to be 94.6%, which is similar to that reported by S. A. Brown et al. (1991). Ceftiofur sodium is reported to have nearly a 100% bioavailability (S. A. Brown et al., 1991) and the kinetics between ceftiofur sodium and ceftiofur hydrochloride are similar (Pharmacia and Upjohn, 1998). Therefore, bioavailability of ceftiofur hydrochloride should have little impact on the kinetics data provided here.

Protein binding in blood of weak acids, such as FLU and CEF, is primarily associated with albumin (Riviere, 2009a). Although we did not see a difference in FLU and CEF pharmacokinetics in this trial, additional work is needed to further characterize drug distribution and protein binding in ill animals. Animals that experience an inflammatory process undergo hepatic down-regulation of albumin synthesis, which spares amino acids for acute phase protein synthesis (Ceciliani et al., 2012), thus reducing available plasma protein binding sites for drugs. In health, protein binding of a drug prevents movement across membranes and into ISF; however, during inflammation, plasma proteins exude into inflamed tissues, potentially causing an increase in the volume of distribution of highly protein bound drugs. The degree of affinity a drug has for a protein will dictate its biological function (Lees, 2009). Protein-bound CEF metabolites are not

biologically active but dissociate quickly to form active metabolites in the chemically reduced environments found in sites of inflammation, (Clarke et al., 1996).

This trial employed three time per day milking schedule. According to the National Animal Health Monitoring System Dairy 2014 survey, 10.2% of herds that participated in the survey milked cows 3x/day. However, 56.8% of herds with 500 cows or greater used this practice (USDA, 2016a). Three times per day milking is utilized by herds as a mechanism to increase milk production per cow (Smith et al., 2002). Several studies have looked at drug clearance from milk following IMM infusion and determined that either increased frequency of removal of milk or increased production of milk per day, as a result of utilizing 3x versus 2x milking, are associated with increased clearance of the drug from the udder (Whittem, 1999; Knappstein et al., 2003; Stockler et al., 2009). The enhanced clearance would also be expected with drugs administered via the parenteral route as lipid solubility and dissociation constant (pKa) of the drug, which are responsible for allowing drugs to move into milk, are not impacted by milking frequency (Ziv and Sulman, 1975). Therefore, the process of establishing equilibrium of the non-ionized fraction of a drug between blood and milk will result in a higher clearance of drug following parenteral administration in cows milked 3 times per day.

In conclusion, the results of this trial indicate that drug interactions between FLU and CEF do not occur when the two drugs are administered simultaneously in healthy cattle. Future work needs to look more closely at CEF movement into diseased tissues and the implications this may have on treatment efficacy and antimicrobial residues.

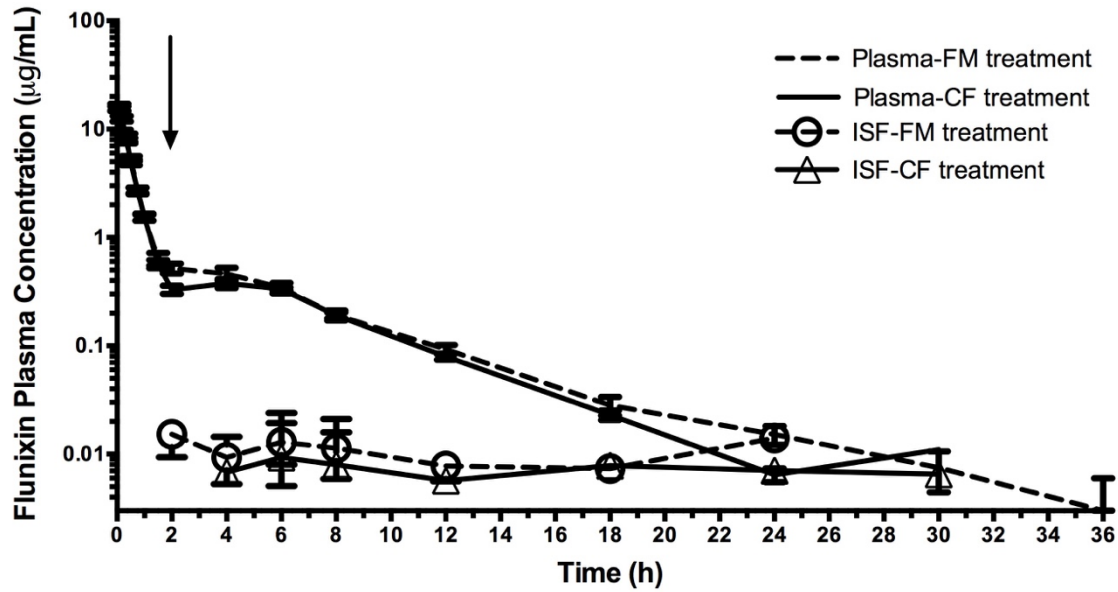


Figure 1. Semi-logarithmic transformations of mean plasma and interstitial fluid flunixin meglumine concentration (± 1 SE) for ten healthy cows following a single IV administration of flunixin compared to a single IV dose of flunixin and a single IM dose of ceftiofur administered simultaneously. Means within time points that differ ($P < 0.05$) are represented by the downward arrow. The x-axis represents the limit of detection for the assay.

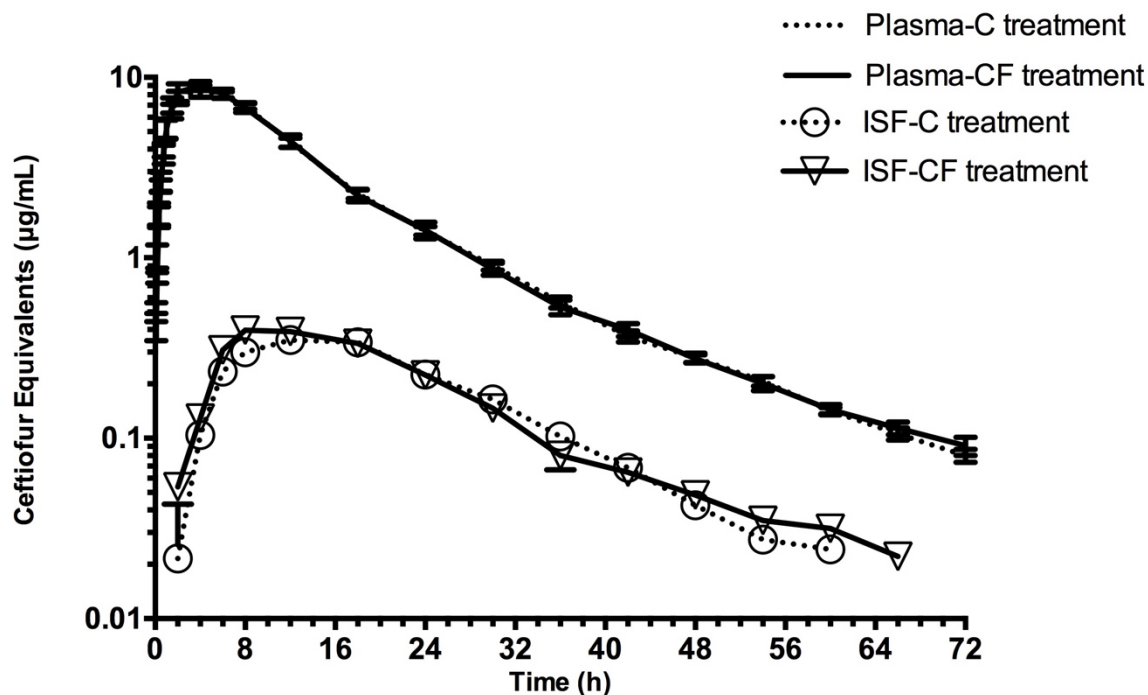


Figure 2. Semi-logarithmic transformations of mean plasma and interstitial fluid ceftiofur equivalents concentration (± 1 SE) for ten healthy cows following a single IM administration of ceftiofur hydrochloride compared to a single IV dose of flunixin and a single IM dose of ceftiofur hydrochloride administered simultaneously. There were no significant differences found between any of the data points. The x-axis represents the limit of detection for the assay.

Table 1. Plasma pharmacokinetic parameters for flunixin meglumine for ten cows that received a single IV dose of flunixin meglumine only compared to a single IV dose of flunixin meglumine and a single IM dose of ceftiofur hydrochloride. Results are presented as arithmetic mean \pm SE.¹

Parameter	Flunixin only treatment	Ceftiofur & flunixin treatment
λ_z (h ⁻¹)	0.18 \pm 0.02 (0.17)	0.19 \pm 0.02 (0.17)
$T_{1/2 \lambda_z}$ (h)	4.49 \pm 0.65 (4.14)	4.92 \pm 1.44 (4.08)
C_0 (μ g/mL)	22.62 \pm 1.06 (22.40)	20.52 \pm 2.22 (19.6)
$AUC_{0-t_{last}}$ (h \times μ g/mL)	10.80 \pm 0.74 (10.57)	10.32 \pm 0.41 (10.25)
$AUC_{0-\infty}$ (h \times μ g/mL)	10.88 \pm 0.74 (10.65)	10.39 \pm 0.40 (10.32)
V_{area} (mL/kg)	547.69 \pm 36.28 (537.68)	560.26 \pm 47.43 (542.83)
CL (mL/h/kg)	217.10 \pm 15.48 (212.54)	225.20 \pm 13.23 (221.84)
MRT (h)	2.58 \pm 0.18 (2.53)	2.48 \pm 0.15 (2.45)

¹Values in parentheses are the geometric mean.

λ_z = slope of the terminal phase; $T_{1/2 \lambda_z}$ = terminal half-life; C_0 = the initial concentration immediately after drug administration calculated by back-extrapolating from the straight line model fit to the first two measured concentrations following IV administration;

$AUC_{0-t_{last}}$ = area under the curve from T0 to the last detectable concentration; $AUC_{0-\infty}$ = area under the curve extrapolated to infinity using the equation $\frac{C_{last}}{\lambda_z}$; V_{area} = volume of distribution based on the terminal phase of the time-concentration curve; CL = clearance; and MRT = mean residence time.

Table 2. Plasma pharmacokinetic parameters for ceftiofur for ten cows that received a single IM dose of ceftiofur hydrochloride only compared to a single IV dose of flunixin meglumine and a single IM dose of ceftiofur hydrochloride. Results are presented as arithmetic mean \pm SE.¹

Parameter	Ceftiofur treatment only	Ceftiofur & flunixin treatment
λ_z (h ⁻¹)	0.05 \pm 0.003 (0.05)	0.05 \pm 0.003 (0.05)
$T_{1/2 \lambda_z}$ (h)	13.42 \pm 0.72 (13.23)	13.96 \pm 0.75 (13.78)
T_{max} (h)	4.0 \pm 0.67 (3.52)	3.2 \pm 0.44 (2.94)
C_{max} (μ g/mL)	9.42 \pm 0.62 (9.26)	10.46 \pm 0.78 (10.24)
$AUC_{0-tlast}$ (h \times μ g/mL)	129.90 \pm 5.24 (129.0)	139.62 \pm 6.62 (138.25)
$AUC_{0-\infty}$ (h \times μ g/mL)	131.48 \pm 5.28 (130.56)	141.54 \pm 6.72 (140.15)
V_{area}/F (mL/kg)	352.84 \pm 28.97 (341.62)	332.91 \pm 24.40 (324.86)
CL/F (mL/h/kg)	18.14 \pm 0.99 (17.90)	16.58 \pm 0.96 (16.34)
MRT (h)	14.03 \pm 0.15 (14.03)	13.91 \pm 0.41 (13.86)

¹Values in parentheses are the geometric mean.

λ_z = terminal elimination rate constant; $T_{1/2 \lambda_z}$ = terminal half-life; T_{max} = time of C_{max} ; C_{max} = maximum plasma concentration; $AUC_{0-tlast}$ = area under the curve from T0 to the last detectable concentration; $AUC_{0-\infty}$ = area under the curve extrapolated to infinity using the equation $\frac{C_{last}}{\lambda_z}$; V_{area}/F = volume of distribution per fraction of the dose absorbed; CL/F = clearance per fraction of the dose absorbed; and MRT = mean residence time.

CHAPTER 4

COMPARATIVE PLASMA AND INTERSTITIAL FLUID PHARMACOKINETICS AND TISSUE DISPOSITION OF CEFTIOFUR CRYSTALLINE FREE ACID IN CATTLE WITH INDUCED COLIFORM MASTITIS

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Abstract

Ceftiofur (CEF) is a third-generation cephalosporin that is the most widely used antimicrobial in the dairy industry. Currently, violative meat residues in cull dairy cattle are commonly associated with CEF. One potential cause for violative residues is altered pharmacokinetics of the drug due to disease, which could increase the time needed for the residue to deplete. The objectives of this study were: 1) to determine the absolute bioavailability of CEF crystalline free acid (CFA) in healthy versus diseased cows; 2) to compare the plasma and interstitial fluid pharmacokinetics and plasma protein binding of CEF between healthy dairy cows and those with disease; and 3) to determine the CEF residue profile in tissues of diseased cows. For this trial, disease was induced through intramammary *E. coli* infusion. Following disease induction and CEF CFA administration, for plasma concentrations, there was not a significant effect of treatment ($P=0.068$) but the treatment by time interaction ($P=0.005$) was significant. There was a significantly greater concentration of CEF in the plasma of the DIS cows at T2 hours ($P=0.002$), T8 hours ($P<0.001$), T12 hours ($P=0.001$), and T16 hours ($P=0.002$). For PK parameters in plasma, the slope of the terminal phase of the concentration versus time curve was significantly lower ($P=0.007$), terminal half-life was significantly longer ($P=0.014$), and apparent volume of distribution during the elimination phase was significantly higher ($P=0.028$) in

the disease group. There was no difference in plasma protein binding of CEF and interstitial fluid pharmacokinetics. None of the cows had kidney CEF residues above the US tolerance level following observation of the drug's withdrawal period but one cow with a larger apparent volume of distribution and longer terminal half-life had tissue residues slightly below the tolerance. While these findings do not support the hypothesis that severely ill cows need longer withdrawal times, alterations in the terminal half-life suggest that it is theoretically possible.

Key words: ceftiofur crystalline free acid, pharmacokinetics, drug residues, dairy cattle

Introduction

Ceftiofur (CEF) is a third-generation cephalosporin antimicrobial that is the most widely used in the dairy industry due to its broad spectrum of activity and short withdrawal periods for milk and meat (Zwald et al., 2004; Swant et al., 2005; Schuler et al., 2017). Broad spectrum antimicrobials are important for both human and veterinary medicine, leading the World Health Organization to classify 3rd, 4th, and 5th generation cephalosporins amongst the highest priority critically important antimicrobials for the preservation of human health (WHO, 2017). Additionally, due to concerns about the development of antimicrobial resistance to cephalosporins in humans from use in farm animals, extra-label usage of CEF was restricted in major food animal species by the United States Food and Drug Administration (US FDA, 2012b).

In CY2013 violative CEF residues became the most frequent residue found in cull dairy cattle at slaughter, surpassing penicillin. Since then, CEF has continued to be the most frequent violative residue in the tissues of cull dairy cattle United States Department

of Agriculture (USDA, 2017b). The reason for this increase in violative ceftiofur residues is likely multi-factorial, including changes to the USDA testing programs that have been implemented over the years (USDA, 2012b), producers unintentionally marketing cattle before the meat withdrawals have elapsed due to record keeping errors, producers intentionally marketing cattle before the meat withdrawals have elapsed to avoid losses from animal death, and potentially alterations in drug metabolism in ill animals compared to healthy counterparts. During the drug approval process, sponsoring companies must present the FDA's Center for Veterinary Medicine (CVM) with toxicological and residue depletion studies. Based on these data, the FDA CVM establishes withdrawal periods for meat and milk, if approved for lactating dairy cattle. However, these studies are performed on healthy animals, not animals suffering from infectious diseases. Data examining drug metabolism in sick animals and how this compares with healthy animals are deficient in the veterinary literature. As most veterinary drugs are not intended for use in healthy animals, data on drug metabolism in diseased animals would provide veterinarians with evidence to more accurately prescribe veterinary drugs and to better predict residue depletion in these diseased animals.

Previous research by the authors recently demonstrated that CEF pharmacokinetics (PK) are altered in dairy cows affected with naturally occurring mastitis compared to healthy cattle (Gorden et al., 2016). In that study, plasma terminal half-life ($T_{1/2 \lambda z}$) of the diseased group was not statistically different from the control group; however, one of the cows in the diseased group had a $T_{1/2 \lambda z}$ that was nearly twice as long as the mean of the control group (70.9 hours vs. 35.8 hours). In that study, animals were not sacrificed upon completion of the study, so tissue residue concentrations were not determined. However,

assuming tissue residue depletion follows plasma PK, this doubling of the $T_{1/2 \lambda_z}$ would indicate that it would take twice as long for the tissues to deplete to the tolerance as would be the case in healthy animals (Riviere et al., 1998). This would necessitate an extension of the withdrawal time by the prescribing veterinarian, even if the drug is used in an on-label manner for dose, duration, and route of administration.

In our previous study (Gorden et al., 2016), the apparent volume of distribution during the elimination phase (V_z/F) and apparent systemic clearance (CL/F) values for CEF were significantly elevated in diseased group. This alteration has also been reported in swine with porcine respiratory and reproductive virus (PRRSv) that were treated with CEF (Tantituvanont et al., 2009; Day et al., 2015; Sparks et al., 2017). In studies where drugs are not administered via intravenous injection, alterations in bioavailability (F) confound the interpretation of V_z and CL . None of the swine experiments or the previous study included an IV administration component to directly determine F of CEF, but Sparks et al. (2017) estimated a relative bioavailability of 0.8. When relative bioavailability was inputted into V_z/F and CL/F parameters for their study, the differences in these two parameters decreased between the control group and animals challenged with PRRSv (Sparks et al., 2017). Therefore, determination of CEF bioavailability will help determine the underlying cause for changes in V_z and CL .

The objectives of this study were to: 1) complete an IV study using CEF sodium to later determine the absolute bioavailability of CEF administered as CEF crystalline free acid (CFA) sterile suspension via a subcutaneous route in healthy versus diseased animals; 2) compare the plasma and interstitial fluid (ISF) concentrations; plasma protein binding; and plasma and ISF PK of CEF following administration as CEF CFA to healthy dairy

cows versus those with disease; and 3) determine the CEF residue profile in kidney, liver, muscle, and fat of diseased cows. In this trial, disease was induced via the administration of *Escherichia coli* via the intramammary (IMM) route. Specifically, we desired to induce the same degree of severity in the disease (DIS) group of animals to mimic the PK profiles of our previous study (Gorden et al., 2016). Our hypothesis was that administration of CEF would result in altered plasma and ISF concentrations, and altered PK in diseased animals compared to healthy animals, necessitating variance in dose regimens and/or withdrawal periods.

Materials and Methods

Experimental cattle

This study was completed at the Iowa State University Dairy Farm. The lactating herd consists of approximately 400 animals (approximately 90% Holstein and 10% Jersey), with 365-day rolling herd averages per cow of 10,991 kg milk, 404 kg fat, and 342 kg protein. Twenty healthy Holstein cows were utilized in two separate segments to complete the objectives of the trial. Ten cows were assigned to the DIS group and ten cows of similar age and lactation status were assigned to the control group (CON). In the first segment, all 20 cows received intravenous CEF as CEF sodium to obtain data to later calculate bioavailability of subcutaneously administered CEF as ceftiofur crystalline free acid (CEF CFA). Segment 2 consisted of the IMM challenge to determine CEF concentrations, PK, and residue depletion. Due to the availability of housing, each segment of the trial was done in two consecutive replicates. The trial was carried out as a 1-sequence, 2-treatment, 2-period cross over design, as the cows that would later be challenged with mastitis were

to be sacrificed at the end. See Figure 1 for a diagram explaining the chronological flow of the trial. Cows were eligible for the trial if they had not been treated with systemic or IMM CEF within the past 20 days of the first segment and were healthy prior to enrollment. Furthermore, the cows were thirty or more days from their next scheduled dry period.

During each treatment segment, cows were housed in individual box stalls bedded with deep, long-stem straw. Each stall had individual access to feed and water. Cows were milked three times daily (4 am, 12 pm, and 8 pm). During the treatment periods, trial personnel milked trial cows per the farm's milking protocol. Between treatments, cows were housed in a free-stall barn bedded with recycled manure solids, which is standard practice for this dairy operation.

Throughout the entire period of this trial, cows were fed a total mixed ration and watered, *ad libitum*. Ration parameters met or exceeded those recommended by the NRC guidelines (NRC, 2001). Cow housing and management met or exceeded the recommendations listed in the *Guide for Care and Use of Agricultural Animals in Research and Teaching* (FASS, 2010). Iowa State University's Institutional Animal Use and Care Committee approved the research protocol prior to commencement of trial procedures (protocol number 6-15-8030-B).

Experimental design – Segment 1 - Ceftiofur bioavailability

One day prior to treatment, cows were weighed and moved to their box stall. One intravenous catheter was placed in each jugular vein of all cows to facilitate CEF administration and blood collection. Following restraint in a stanchion, cows were sedated with xylazine at approximately 0.025 mg/kg IV; the skin over the jugular furrow was

clipped and aseptically prepped using alternating scrubs of 2% chlorhexidine acetate and 70% isopropyl alcohol. Prior to catheter placement, the area under the skin was infiltrated with 2% lidocaine. Following catheter placement, the catheter was sutured in place using #3 nylon suture. To maintain catheter patency, 3 mL of a heparin saline solution containing 3 USP units of heparin sodium/mL was infused into the catheters every eight hours until treatments were initiated. Subsequently, catheters were flushed following each blood collection.

On the day of treatment, cows were restrained in a stanchion, where cows received CEF as ceftiofur sodium (Naxcel Sterile Powder, Zoetis Inc., Kalamazoo, MI) at a dose of 2.2 mg/kg via the IV route. Following CEF administration, 3 mL of a heparin saline solution was infused into the catheters to assure complete delivery of the drug. The catheter used for CEF administration was then removed.

At T0 hour prior to CEF administration, two 10-mL blood samples were collected from the jugular catheter into blood tubes containing freeze-dried heparin (Becton, Dickinson and Co, Franklin Lakes, NJ) for plasma harvest. Subsequent blood samples were collected from the jugular catheter into heparinized tubes at 0.05, 0.10, 0.25, 0.5, 0.75, 1, 2, 4, 6, 8, 16, 24, 32, 40, and 48 hours after drug administration. After blood was collected, samples were immediately placed on ice until plasma could be harvested. Within two hours of collection, blood samples were centrifuged for 20 minutes at 1000 g at 4 °C, then 5 mL of plasma was harvested and frozen at -70°C until analyzed for drug concentration.

Following the 48 hour time point, IV catheters were removed and the cows were moved back to the free stall housing for the herd. The cows had a minimum of a ten-day

washout period between the two segments of this trial. Both replicates of this segment of the trial were completed prior to initiation of the second segment of the trial.

Experimental design – Segment 2 – Pharmacokinetics and tissue residue depletion of CEF CFA in healthy versus diseased cows

One day prior to treatment, cows were moved to box stalls and had one IV catheter inserted as described above. While sedated, all cows had one subcutaneous, *in vivo* ultrafiltration probe (RUF 3-12, BASi, West Lafayette, IN) placed dorsal-caudal to the scapula to facilitate ISF collection. Briefly, the area was prepped as described for inserting catheters above and the probe was placed by passing a 10-gauge metal introducer needle between two small stab incisions previously made with a #10 scalpel blade into the skin. Following probe placement, the collection tube was stitched in place and connected to a 7-mL red top glass vacuum tube (Becton, Dickinson and Co, Franklin Lakes, NJ) for ISF collection.

Intramammary Challenge

To induce disease, cows in the DIS group were inoculated with 100-150 colony forming units (cfu) of *E. coli* (strain 487) via the streak canal of a selected mammary gland quarter. To prepare the challenge inoculum, an aliquot of frozen stock culture was streaked onto a trypticase soy agar plate and incubated overnight at 37 °C. The following day, two well isolated colonies were inoculated into trypticase soy broth and incubated overnight in a shaker incubator at 37 °C to achieve stationary growth phase. Two-mL aliquots of the broth culture were centrifuged in microcentrifuge tubes and the pellet was washed twice in

phosphate buffered saline (PBS) and then re-suspended in PBS to achieve an optical density of 0.35 at 495 nm. Seven 9:1 serial dilutions were then made in PBS and 0.1 mL was plated on trypticase soy agar and incubated overnight. The following day, cfu counts were enumerated. The process was then completed to acquire a challenge inoculum with a desired cfu count of 100-150 diluted in 5 mL of PBS.

Approximately 12 hours prior to CEF administration (T -12 hours), all cows in the DIS group were inoculated with the challenge inoculum in either the right or left front quarter following aseptic preparation of the teat end. Simultaneously, cows in the CON group underwent a placebo challenge by infusing 5 mL of sterile PBS into the right or left front quarter following aseptic preparation of the teat end. The decision on whether to challenge the left or right front quarter was based on position of the dividing gate between stalls. Therefore, the challenged quarter was always opposite from the dividing gate.

Following completion of the IMM challenge, 0.1 mL of challenge inoculum was plated onto trypticase soy agar and incubated overnight to determine *E. coli* challenge dose.

Drug administration

At time 0 (T0), all cows received CEF, as CEF CFA (Excede, Zoetis Inc., Kalamazoo, MI), at 6.6 mg CEF equivalents per kg of body weight administered at the base of either ear, following instructions on the package insert. As part of the trial design, rescue therapies (anti-inflammatories and fluid support) were included in the trial protocol if needed. No further medications were administered throughout the remainder of the trial.

Collection of blood and ISF samples

Prior to CEF administration (T0), two 10-mL blood samples were collected from the jugular catheter into blood tubes containing freeze-dried heparin (Becton, Dickinson and Co, Franklin Lakes, NJ) for plasma harvest. Subsequent blood samples were collected from the jugular catheter into heparinized tubes at 2, 4, 8, 12, 16, 20, 24, 32, 40, and 48 hours and then every 24 hours after drug administration through 312 hours. After blood was collected, samples were processed as described above.

Simultaneous with drug administration (T0), a new vacuum tube was attached to the ultrafiltration probe. Interstitial fluid samples were collected at 4, 8, 12, 16, 20, 24, 32, 40, and 48 hours following drug administration and then every 24 hours through 312 hours, by changing the vacuum tube. The tubes were immediately frozen at -70 °C until analyzed for drug concentration.

Daily observations and infrared thermography

At every milking for the first five days following IMM challenge and then daily through the conclusion of the trial, cows were assigned a mastitis severity score as previously described by Wenz et al. (2001b). Additionally, rectal temperatures were recorded at T0 and then every 8 hours for the first 24 hours and then every 24 hours for the remainder of the trial.

Infrared images of the eye on the side of CEF CFA injection, the ear where the CEF CFA injection was placed, and quarter of the mammary gland that was challenged were obtained using a research quality infrared camera (FLIR SC 660, FLIR Systems, AB, Danderyd, Sweden). Images were obtained prior to IMM challenge (T -12 hours), at T0

prior to CEF CFA injection, at 8, 16, and 24 hours following injection, and then every 24 hours through T168 hours. At each measurement period, three images each (nine total) were collected from the eye, ear, and mammary gland, respectively. Images of the eye and ear were obtained by holding the camera at approximately a 45° angle and 0.5 meters from the head. Mammary gland images were collected by placing the camera in a parallel plane lateral to the challenged quarter, approximately 0.5 meters from the gland. Camera calibration was done prior to each measurement period by entering current ambient temperature and relative humidity into the camera's software. Throughout each measurement period, the camera collected changes to ambient temperature and relative humidity and recalibrated automatically.

Analysis of infrared images was completed using research grade software provided by the camera manufacturer (FLIR ExaminIR, North Billerica, MA). For each measurement period, the maximum, minimum, and mean temperature was recorded for each image and a mean value for each the parameters (maximum, minimum, and mean) was determined from the three images for the eye, ear, and mammary gland, respectively.

Trial conclusion

At T312 hours, all DIS cows were humanely euthanized with a captive bolt followed by exsanguination. Following euthanasia, kidney, liver, skeletal muscle, fat, and injection site tissues were collected, weighed, and frozen at -70 °C until analyzed for drug concentration.

Cows in the CON group were returned to the herd following catheter and subcutaneous ultrafiltration probe removal.

Determination of plasma protein binding

Bound plasma drug concentration of CEF was determined on each cow on the T24, T96, and T192 hours plasma samples using a microcentrifugation system (Centrifree Ultrafiltration Device, EMD Millipore Corp., Billerica, MA) to collect plasma ultrafiltrate (UF), as previously described (Gorden et al., 2017). Following collection of plasma UF, samples were immediately frozen at -70 °C until analyzed for drug concentration.

Plasma, interstitial fluid, and plasma ultrafiltrate ceftiofur concentration analysis

Ceftiofur and its metabolites from plasma were converted to a stable derivative, desfuroylceftiofur acetamide (DCA) and total CEF concentration (as DCA) was then determined using liquid chromatography coupled with mass spectrometry (LC-MS) as previously described (Gorden et al., 2016). Plasma UF and ISF samples were analyzed for free drug concentration in the same manner, except spike and quality control (QC) samples were prepared using blank ISF for ISF sample analysis. The limit of detection (LOD) of the assay was 1 ng/mL, and the limit of quantification (LOQ) was 10 ng/mL. The accuracy and coefficient of variation for the quality control (QC) samples were 98% and 9.4% for the 15 ng/mL QC sample; 105% and 8.7% for the 150 ng/mL QC sample; and 107% and 10.6% for the 1,500 ng/mL QC sample.

Screening of kidney samples – Kidney Inhibition Swab (KIS™) test

At tissue harvest, a section of kidney from each cow was bagged and frozen separately. After at least 24 hours of freezer storage, all ten samples were thawed at 4 °C

and tested for inhibitory residues using the Kidney Inhibition Swab (KIS™) test (Charm Sciences Inc., Lawrence, MA) as described (USDA, 2010). Additionally, kidney tissue from a negative control animal was also thawed and tested. Briefly, the cap portion of the swab was used to cut a circular incision into the kidney parenchyma approximately 1-2 cm in depth. The swab was then placed into the incised area and rotated for approximately 30 seconds to saturate the swab with kidney fluid. This procedure was repeated on up to four swabs in total at one time. The swab was then pierced through the foil and into the clear liquid in the bottom vial of the test, but not perforating the bottom seal. After two minutes, the swab was completely screwed down to pierce the bottom seal and to the point where it was just above the agar in the bottom of the vial. The tube was then tapped firmly five times on the countertop, after which the swab was rotated in the opposite direction and tapped five times again on the countertop. Up to four swabs were then placed into the heating block provided with the test kit and incubated at 64 °C for three hours. Following incubation, the agar color was compared to the reference card provided by the manufacturer to determine the test result.

Ceftiofur concentration analysis-tissue samples

Determination of ceftiofur concentrations in kidney tissues was completed using an official method as described by the USDA (2016c), with minor alterations to the protocol. The method determines the concentration desfuroylceftiofur cysteine disulfide (DCCD) as a proxy for the marker compound for ceftiofur, desfuroylceftiofur (DFC). Briefly, 0.4 g aliquots of blank bovine kidney for blank, spike, fortified analyst recovery, and QC samples; in addition to 0.4 g aliquots of test kidney samples, were weighed into 15-mL

conical bottom, polypropylene tubes. Known concentration of DCCD were added to spike kidney samples to create a calibration curve from 50 – 2000 ng/g. 5000 ng of internal standard, DCCD-d3, was then added to all spike, QC, blank, and test samples but not the fortified recovery analyst tube. All tubes were treated with 3.5 mL of 1% phosphate buffer and shaken on an automated shaker for ten minutes at 1000 rpm. Following shaking, all tubes were centrifuged at ~4000 rpm for 20 minutes at room temperature. Solid phase extraction (SPE) cartridges (Strata-X SPE cartridges (60 mg/3 mL), Phenomenex, Torrance, CA, USA) were conditioned with 2 mL of methanol, followed by 2x2 mL fractions of ultrapure water. The supernatant following centrifugation was then loaded onto the SPE cartridges and allowed to percolate via gravity. After all the supernatant had percolated through the SPE cartridges, they were washed with 2 mL ultrapure water. Target analytes were eluted with 2x1 mL fractions of 50% acetonitrile/ultrapure water (v/v). Following elution, the fortified analyst recovery tube was spiked with sufficient DCCD to create a concentration of 400 ng/g and 5000 ng of the internal standard, DCCD-d3. The acetonitrile was then evaporated from the samples under a stream of nitrogen at 15 psi and 48 °C to a volume of <1 mL. Ultrapure water was added to each sample to bring the total volume to approximately 1 mL and then all samples were vortexed. 150 µl of sample was transferred to labelled auto-sampler vials equipped with glass inserts for analysis by LC-MS. Auto-sampler vials were centrifuged for 20 minutes at 1000 g at room temperature and then loaded onto the auto-sampler tray.

The LC-MS system consisted of an Agilent 1100 pump, auto-sampler, and column compartment (Agilent Technologies, Santa Clara, CA) coupled to an ion trap mass spectrometer (LTQ, Thermo Scientific, San Jose, CA). The injection volume was set to 25

μL. The mobile phases consisted of A) 0.1% formic acid in water and B) 0.1% formic acid in acetonitrile at a flow rate of 0.25 mL/min. The mobile phase began at 5% B with a linear gradient to 95% B at 6.5 minutes, which was maintained for 1.75 minutes, followed by re-equilibration to 5% B. Separation was achieved with an ACE C18 column (ACE 3 C18, 150 mm × 2.1 mm, 3 μm particles, Mac-Mod Analytical, Chadds Ford, PA, USA) maintained at 40 °C. DCCD was eluted at 3.63 minutes and the internal standard, DCCd-d3, eluted at 3.61 minutes.

Sequences consisting of plasma blanks, calibration spikes, quality control samples, fortified analyst recovery, and bovine kidney samples were then batch-processed with an automated processing method developed in the Xcalibur software (Thermo Scientific, San Jose, CA, USA), which identified and integrated each sample peak. The calibration curve was calculated based on a weighted (1/X), linear fit. Tissue concentrations of DCCD in trial samples were calculated based on this calibration curve. Results were then viewed in the Quan Browser portion of the Xcalibur software. The standard curve had a linear range from 50 to 2000 ng/g, with a correlation coefficient of 0.993. All standards were within +/- 15 % of the nominal value in this range with the exception of the lowest (50 ng/g) standard, which was within + 20% of the nominal value. The accuracies for the QC samples were 91% for the 75 ng/g QC, 106% for the 750 ng/g QC, and 110% for the 1,500 ng/g QC. The accuracy of the fortified analyst recovery sample was 94%. The limit of detection and the limit of quantification for this assay was 50 ng/g.

In the US, the official marker residue for CEF in the bovine is DFC, measured as DCA. In the bovine, the current US tolerance is 0.4 parts per million (400 ng/g), with kidney serving as the marker residue. The method described above has been published as

an alternate method to determining DCA in tissues, in which DCCD is measured as a surrogate marker residue for DFC (Feng et al., 2014). In order to convert measured DCCD concentrations to DFC, the following regression equation was utilized:

$$y = 0.21557 + 1.801 \times x$$

where y = the DFC concentration being calculated and x = the DCCD determined concentration (Feng et al., 2014).

After results from determination of DCCD in kidney tissues were evaluated, other tissue samples were not analyzed.

Pharmacokinetic analysis

The total plasma drug and ISF concentration-time profiles from CEF CFA treated cows were analyzed using non-compartmental methods implemented in a commercially available software program (Phoenix[®] Win-Nonlin[®] 7.0, Certara, Inc. Princeton, NJ) to generate the following PK parameters: λ_z (h^{-1}), slope of the terminal phase; $T_{1/2\lambda_z}$ (h), terminal half-life; C_{\max} ($\mu\text{g/mL}$), maximum plasma concentration; T_{\max} (h), time of C_{\max} ; $\text{AUC}_{0-\infty}$ ($\mu\text{g/mL} \times \text{h}$), area under the curve extrapolated to infinity using the equation $\frac{C_{\text{last}}}{\lambda_z}$; $\text{AUC}_{0-24\text{hr}}$ ($\mu\text{g/mL} \times \text{h}$), area under the curve from T0 to T24 hours; V_z/F (mL/kg), apparent volume of distribution during the elimination phase; and CL/F (mL/h/kg), apparent systemic clearance; and $\text{MRT}_{0-\infty}$ (h), mean residence time extrapolated to infinity using the equation $\frac{C_{\text{last}}}{\lambda_z}$. AUC and MRT were extrapolated to infinity to account for the total exposure to the drug.

Determining the absolute bioavailability for extended release drugs like CEF CFA is often complicated due to flip-flop kinetics as clearance can be affected by the rate of

absorption. To determine if there was a difference in CL when CEF CFA was administered, the absorption rate constant (k_a) was determined first determining the mean absorption time (MAT) using the equation:

$$\text{MAT} = \text{MRT}(\text{SQ}) - \text{MRT}(\text{IV})$$

where $\text{MRT}(\text{SQ})$ = the mean residence time via the subcutaneous route and $\text{MRT}(\text{IV})$ = the mean residence time via the intravenous route. The k_a for each treatment group was then determined using the equation:

$$\text{Absorption rate constant (ka)} = \frac{1}{\text{MAT}}$$

To determine the apparent bioavailability (F) in cows treated with CEF CFA, the $\text{AUC}_{0-\infty}$ was determined from plasma samples collected following IV ceftiofur sodium administration. Bioavailability was then determined and corrected for differences in CL between the two routes of administration using the equation:

$$F(\%) = 100 * \frac{\text{AUC}(\text{SQ}) * \text{D}(\text{IV}) * \text{CL}(\text{SQ})}{\text{AUC}(\text{IV}) * \text{D}(\text{SQ}) * \text{CL}(\text{IV})}$$

where $\text{AUC}(\text{SQ}) = \text{AUC}_{0-\infty}$ determined for CEF CFA via the subcutaneous route; $\text{D}(\text{IV})$ = dose of ceftiofur sodium administered via the IV route; $\text{CL}(\text{SQ})$ = clearance following subcutaneous administration; $\text{AUC}(\text{IV}) = \text{AUC}_{0-\infty}$ determined for ceftiofur sodium via the IV route; $\text{D}(\text{SQ})$ = dose of CEF CFA administered via the subcutaneous route; and $\text{CL}(\text{IV})$ = clearance following IV administration.

Data analysis

Statistical analysis was performed using a commercially available software program (SAS 9.4, SAS Institute, Cary, NC). All data are expressed as arithmetic mean \pm SE and

geometric mean. Comparison of variables between treatment groups that were single observations (e.g., enrollment variables and PK parameters) were made using a paired t-test unless the values were not normally distributed (ISF C_{\max} and $AUC_{0-\infty}$). For these parameters, means were compared using the Wilcoxon two-sample rank-sum test. Drug concentrations in plasma and ISF, protein binding, rectal temperatures, and IRT values for the DIS and CON groups were analyzed via the GLIMMIX procedure using repeated measures, with the animal being the subject of repeated measures. Fixed effects were treatment (DIS or CON), time, and the interaction between treatment and time. Replicate was included as a random effect. Statistical significance was established when $P < 0.05$.

Results

At enrollment, there was no statistical difference for any of the animal enrollment variables between the treatment groups. Between the first and second segments of this trial, two CON cows were removed due to illness that was treated with systemic CEF therapy. Therefore, only the eight remaining cows were used in the calculation of F of CEF CFA in the CON group. The two animals were replaced for the completion of the second segment of the trial, therefore all other parameters have 10 animals per treatment.

Following IMM challenge, *E. coli* concentration in the challenge inoculum was determined to be 105 cfu for replicate 1 and 184 cfu for replicate 2. Following challenge, all cows in the DIS group developed clinical mastitis within 12 hours (by T0 hour). As a result, four of ten cows developed clinical mastitis classified as moderate and the remaining were classified as severe. However, none of the cows developed clinical signs necessitating rescue therapy.

No cow had detectable CEF in plasma or ISF at the beginning of either segment (time 0). Following IV administration of CEF sodium, all cows had measurable CEF in their plasma throughout the 48-hour monitoring period (data not shown). Plasma and ISF concentrations of CEF for cows in segment 2 are displayed in Figure 2. Following CEF CFA administration, CEF was detected in all subsequent plasma samples throughout the entire study period. For plasma concentrations, there was not a significant effect of treatment ($P=0.068$) but the treatment by time interaction ($P=0.005$) was significant. There was a significantly greater concentration of CEF in the plasma of the DIS cows at T2 hours ($P=0.002$), T8 hours ($P<0.001$), T12 hours ($P=0.001$), and T16 hours ($P=0.002$). There were no other time points that were significantly different between the two groups for the remainder of the trial.

For the ISF samples, only eight cows had quantifiable CEF concentrations in their ISF four hours after therapy, but by eight hours, all cows with functional ultrafiltration probes had quantifiable CEF concentrations in their ISF. One cow had a malfunctioning ultrafiltration probe for the first 16 hours after CEF CFA administration. Two cows did not have quantifiable CEF in ISF past 48 hours and seven cows had quantifiable CEF through 192 hours. There was a significant effect for time ($P<0.001$) but treatment and the treatment by time interaction were not significant.

Mean protein binding (± 1 SE) of CEF at T24 hours was $91.1\% \pm 0.93$ for the CON group and $93.0\% \pm 0.93$ for the DIS group. At T96 hours, mean protein binding was $92.5\% \pm 0.93$ and $90.6\% \pm 0.93$ for the CON and DIS groups, respectively; while at T192 hours, the bound fraction was $93.8\% \pm 1.3$ and $94.3\% \pm 1.03$, respectively. There were no significant differences in protein binding between any of the groups.

Plasma pharmacokinetic parameters are shown in Table 1. Between the two groups, λ_z was significantly lower ($P=0.007$), $T_{1/2 \lambda_z}$ was significantly longer ($P=0.014$), and V_z/F was significantly higher ($P=0.028$) in the DIS group. The mean (and range) value for $T_{1/2 \lambda_z}$ was 58.52 (44.82-80.37) hours for the DIS group and 45.87 (39.05-58.01) hours for the CON group. For V_z/F , the mean (and range) of value for the CON group was 1.745 (1.230-2.146) L/kg while the DIS group was 2.135 (1.574-2.910) L/kg.

Comparisons of λ_z following IV administration to calculated k_a are presented in Table 2. The k_a/λ_z ratio was 0.16 for the CON group and 0.25 for the DIS group, indicating the CEF CL following subcutaneous CEF CFA administration was much lower than following IV administration. Additionally, the mean difference between the k_a/λ_z ratios was significant ($P=0.003$), indicating CL in the DIS group was impacted by disease. The mean apparent bioavailability was significantly lower in the DIS group ($P<0.001$). The mean apparent bioavailability in the DIS group was 98.75% while the mean in the CON group was 110%.

There were no other statistically significant differences between any of the PK parameters.

Interstitial fluid PK parameters are displayed in Table 3. There were insufficient samples from three cows (2 CON and 1 DIS) to determine all the PK variables, so their data was excluded. There were no statistically significant differences between any of the ISF PK parameters between the two groups.

All kidney KIS tests were negative. Kidney DCCD concentrations in are presented in Table 4. Only two cows had DCCD concentrations in kidney tissue above the LOQ for the assay. When converted to DFC, both cows had concentrations below the US tolerance

for CEF in bovine kidney tissues. As none of the kidney tissues had violative kidney residues, the remaining tissues were not analyzed.

There was no effect of treatment or time by treatment interaction on rectal temperature; however, time had a significant effect ($P < 0.001$). Specifically, T0 hour ($P < 0.001$) and T8 hours ($P = 0.031$) were significantly elevated, while T288 ($P = 0.004$) and T312 hours ($P < 0.001$) were lower than the mean temperature. Thermography was evaluated at the base of the ear at the injection site, on the ipsilateral eye, and on the challenged quarter of mammary gland. For ear images, there were no significant treatment, time, or treatment by time interactions between the two groups, except the time variable for maximum ($P = 0.016$) and minimum ($P = 0.012$) temperature. For eye images, the time variable for maximum, minimum, and average temperatures were significant ($P < 0.001$). There was no effect for treatment or the interaction of treatment by time for eye images. There were also significant time differences for maximum ($P = 0.033$), minimum ($P < 0.001$), and average ($P = 0.009$) mammary gland temperatures, but not treatment or treatment by time interaction.

Discussion

In this trial, no cow required rescue therapy as a consequence of their illness. Compared to previous work (Gorden et al., 2016), the cows in the current trial did not get as clinically ill and the duration of illness was shorter. Additionally, all cows continued lactating as compared to the previous work where five of eight cows developed agalactia as a result of their illness.

Plasma C_{\max} concentrations of CEF following administration of CEF CFA were approximately equal to those published for dairy cattle in the package insert and lower than

those for beef cattle from the package insert (Zoetis Inc., 2013b). C_{\max} values were also lower for a beef cattle study published by others (Washburn et al., 2005). Time to maximum concentration was approximately four hours shorter in this work compared to the package insert, the $AUC_{0-\infty}$ was slightly lower in this work, and the $T_{1/2}$ in the CON group was approximately equal to the package insert (Zoetis Inc., 2013b).

Initially, the cows in the DIS group had a numerically higher CEF plasma concentration, which persisted through 40 hours post-treatment. This phenomenon was also present in our previous work for approximately ten hours after the first dose of CEF hydrochloride (Gorden et al., 2016). While C_{\max} in this trial was not determined to be significantly higher in the DIS group, there was a tendency for a higher C_{\max} ($P=0.081$). It is plausible that the febrile response associated with the clinical mastitis in the DIS group could have resulted in more blood flow to the injection site as previously described by Groothuis et al. (1978, 1980), resulting in numerically higher plasma drug concentrations early in the course of disease.

Additionally, the cows in this trial had saw tooth-like DCA plasma concentrations that continued until approximately 32 hours after treatment. This was also noted in the previous work on individual cows, but was not apparent on the mean concentration graph (Gorden et al., 2016). Desfuroylceftiofur is reported to have a lower initial volume of distribution than CEF (Whittem et al., 1995), which will likely account for the up and down pattern of plasma concentrations as CEF is absorbed from the injection site. Additionally, altered hepatic metabolism of parent CEF to the DFC metabolite in the DIS group could have contributed in differences in plasma concentration over time between groups. In segment 1 of this trial, plasma DCA concentrations following IV administration of CEF

increased from T0.05 to T0.5 hours (data not shown). This phenomenon has previously been described and is apparently the result the lower initial volume of distribution of DFC compared to its parent compound (Whittem et al., 1995).

Data from the IV CEF study was used to calculate the absolute bioavailability of CEF administered as CEF CFA. In both groups, F was determined to be approximately 160%, which is a reason for concern as bioavailability values >100% are theoretically implausible in cases of linear clearance (Toutain and Bousquet-Mélou, 2004). There are three potential issues at hand that could contribute to this phenomenon. First, this trial was conducted as a 1-sequence, 2-treatment, 2-period crossover design, as compared to the recommended 2-sequence crossover approach when completing bioavailability trials. This potentially introduces the risk of having a period effect and/or a carry-over effect from the first period to the second on the exposure estimates. Another consideration is that the extrapolated portion of $AUC_{0-\infty}$, from the last plasma measurement to infinity could make up a large portion of the estimated AUC value. However, in this trial, the extrapolated portion was <2.5% for all $AUC_{0-\infty}$ determinations. The third, and likely explanation for the elevated bioavailability value is due to changes in CL associated with extravascular administration. Following administration of a drug exhibiting flip-flop kinetics, values for λ_z for the IV route and k_a (termed $k_{a,fl}$ in the remainder of the manuscript) should be approximately equal if CL is unaffected. However, in this trial the value for $k_{a,fl}$ was 16% and 25% of the value for λ_z in the CON and DIS groups, respectively. As $k_{a,fl}$ is substantially lower, the CL following subcutaneous administration of CEF CFA is lower than the CL following IV administration. This results in increased exposure of drug following the subcutaneous administration and an elevated AUC(SQ), which resulted in a bioavailability

value that was much greater than 100%. Insertion of IV and subcutaneous CL values into the bioavailability equation corrected for the lower subcutaneous CL, resulting in an apparent bioavailability value closer to 100% for both treatment groups. Interestingly enough, in an equine trial using a parallel design versus IV CEF sodium, the absolute bioavailability of CEF CFA has been reported to be 100%, with a 90% confidence interval ranging from 92.4 to 109% (Collard et al. 2011), supporting our findings in dairy cattle.

Maximum CEF concentrations in ISF were lower and time to reach maximum concentration was longer in the current trial compared to previous work (Gorden et al., 2017). In addition, CEF was detected in ISF for approximately 120 hours in the current study compared to 60 hours in the previous work. Given that CEF CFA exhibits flip-flop kinetics, this is not surprising. Foster et al. (2015) have also reported ISF PK parameters using similar tissue probes utilized in the current study. In their work, they reported higher ISF CEF C_{\max} , a more rapid T_{\max} , but nearly identical AUC compared to the current work in a study utilizing healthy six-month old Holstein steers administered CEF sodium at 2.2 mg/kg. These C_{\max} and T_{\max} values were similar to the previous work, where ISF CEF concentration following CEF hydrochloride administration was determined (Gorden et al., 2017). Again, differences in kinetics of absorption likely account for these differences. Washburn et al. (2005) also reported lower C_{\max} and a longer T_{\max} using fluid collected from uninfected tissue cages following CEF CFA administration in trials using feedlot animals, compared to earlier work by the same research group when CEF sodium was administered via the IV route (Clarke et al., 1996). However, the C_{\max} and AUC values reported by Washburn et al. (2005) are significantly higher than those reported in our current work or that reported by Foster et al. (2015). Washburn et al. (2005) also observed

even higher CEF C_{\max} and AUC values in tissue cages infected with *Mannheimia haemolytica* compared to uninfected cages. Tissue cage data should be interpreted carefully, as these create an artificial fluid filled space that allows protein to escape the vasculature and enter the tissue cage (Davis et al., 2005). As CEF is reported to be 50-90% protein bound (S. A. Brown et al., 1991), a major portion of the drug represented in the Washburn et al. (2005) would be protein bound and not biologically active. Clarke et al. (1996) state that bound fractions of CEF will dissociate quickly in chemically reduced environments found in areas of inflammation. However, it would seem prudent to utilize tissue probe data to interpret biological function of CEF in ISF.

The fact that none of the kidney inhibition swab (KISTTM) tests were positive on the DIS animals is not surprising given the fact that all the kidney tissues were below the tolerance. Additionally, the reported sensitivity of this assay is 4 ppm, which is 10-fold higher than the tolerance for CEF (Jones et al., 2014), making it a questionable choice for screening cull dairy cattle for CEF residues. This is thought-provoking since cull dairy cows have the highest incidence of violative residues amongst adult cattle classes (USDA, 2017b) and CEF is the most commonly used antimicrobial in the US dairy industry (Zwald et al., 2004; Swant et al., 2005; Schuler et al., 2017). Taken together, it is highly likely that animals with violative residues for CEF are not being submitted for confirmatory testing due to the limited sensitivity of this screening test for CEF.

During the drug approval process, sponsoring companies must present the FDA CVM with toxicological and residue depletion studies. Based on these data, the FDA CVM establishes withdrawal periods for meat and milk, if approved for lactating dairy cattle. However, these studies are performed on healthy animals, not animals suffering from

infectious diseases. In this study, we were able to determine CEF residue levels in kidney tissues following CEF CFA treatment in animals that experienced induced coliform clinical mastitis. Of the ten animals that were challenged, only two had kidney residues for DCCD above the LOQ for the assay. In order to convert measured DCCD concentrations to DFC, a regression equation was utilized (Feng et al., 2014). As a result, one cow was determined to have a DFC residue level of 0.32 $\mu\text{g/g}$ (cow #9389) and another of 0.38 $\mu\text{g/g}$ (cow #9206). These are both below the established tolerance for DFC in kidney tissue of 0.4 $\mu\text{g/g}$ (US FDA, 2006a). When looking at the individual PK parameters for cow #9206, her plasma CEF $T_{1/2 \lambda z}$ (80.3 hours) was the highest of all the DIS cows. This is nearly two times as long as the CON average. Additionally, this cow was the one who suffered the most severe clinical disease based on clinical appearance, rectal temperature, and daily feed refusal. In the previous work (Gorden et al., 2016), a much wider range for V_z , CL , and subsequently $T_{1/2 \lambda z}$ was observed. If tissue residue depletion follows plasma PK, this doubling of the $T_{1/2 \lambda z}$ would indicate that it would take twice as long for the tissues to deplete to the tolerance as would be the case in healthy animals (Riviere et al., 1998). In order to account for variation in tissue depletion amongst animals, the FDA utilizes a process based on the statistical tolerance limit procedure (De Gryze et al., 2007). In applying this procedure to the determination of withdrawal periods for a drug, FDA selects the 99th percentile tolerance limit with a 95% confidence. This should mean that 99% or more of tissue samples are at or below the tissue tolerance in the target tissue when the withdrawal period has elapsed (US FDA, 2006b).

The primary weakness of this trial was not creating the level of illness in DIS group animals as was seen in the previous trial (Gorden et al., 2016). In that trial, five of eight

trial cows developed severe disease and agalactia, despite aggressive supportive therapy. In this work, no cows required rescue therapy. Additionally, the attempts to objectively characterize febrile responses using rectal temperature and IRT measurements to separate out treatment effects between the DIS and CON animals proved to be unsuccessful. In both replicates of segment 2, high ambient temperatures during the first 48 hours of each challenge period likely confounded our ability to assess differences in rectal and IRT temperatures. Future research should focus on identifying clinical parameters that are associated with altered PK parameters, which would allow producers to implement longer withdrawal periods in order to minimize risk of marketing an animal with a violative residue.

In conclusion, the results of this trial support previous work that cows suffering clinical disease associated with mastitis may have altered CEF volume of distribution and terminal half-life. It did not however support the hypothesis that severely ill cows need longer withdrawal times following CEF therapy. However, substantially larger V_z/F and longer $T_{1/2\lambda_z}$ on some cows suggest that this may be possible in a clinical disease in a large population. Future work needs to identify parameters in cattle that should be monitored in order to implement longer withdrawal periods on cows potentially at risk for maintaining violative residues past their withdrawal period.

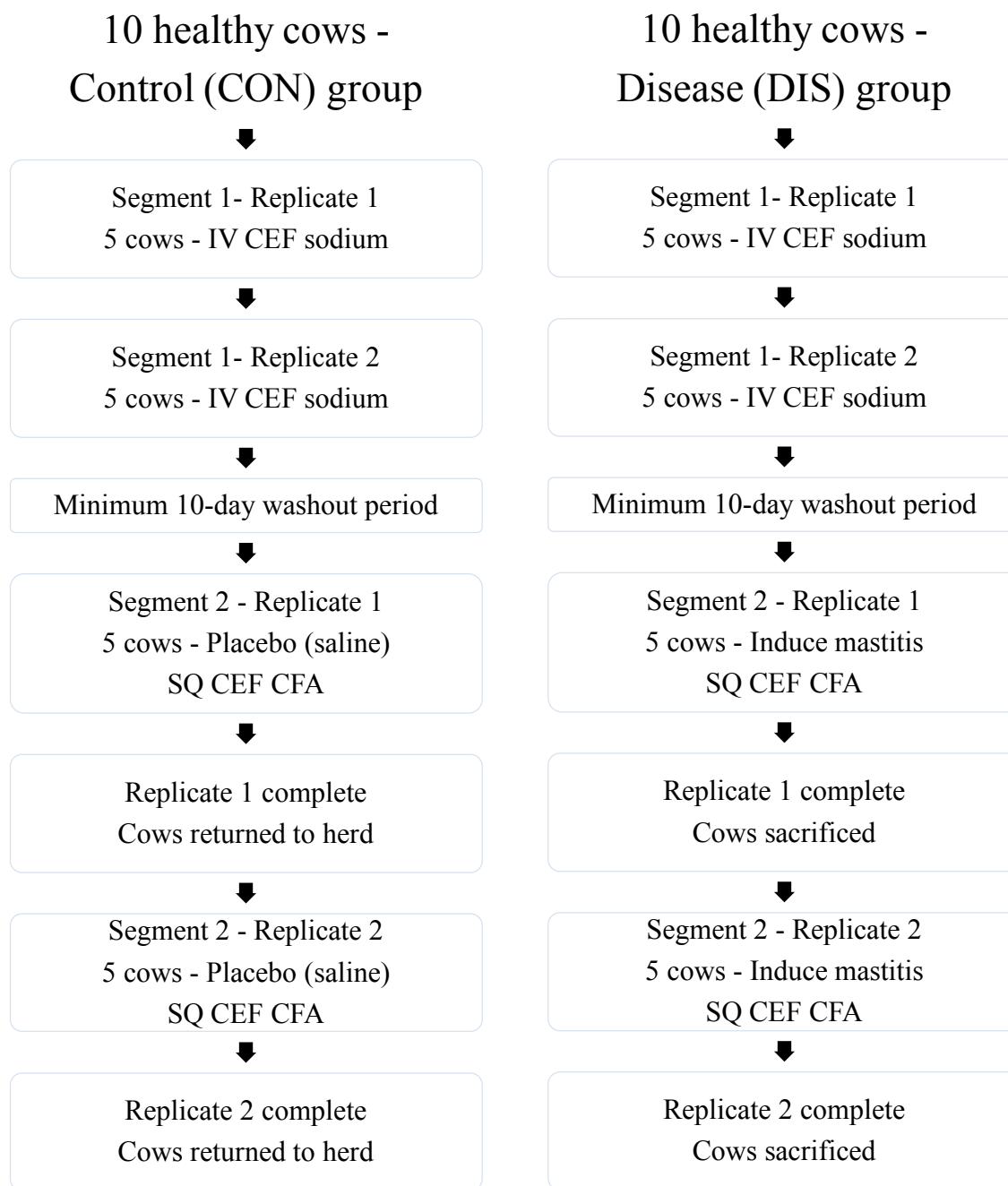


Figure 1. Flow diagram showing the chronological flow of the segments. Boxes in the same row occurred at the same time.

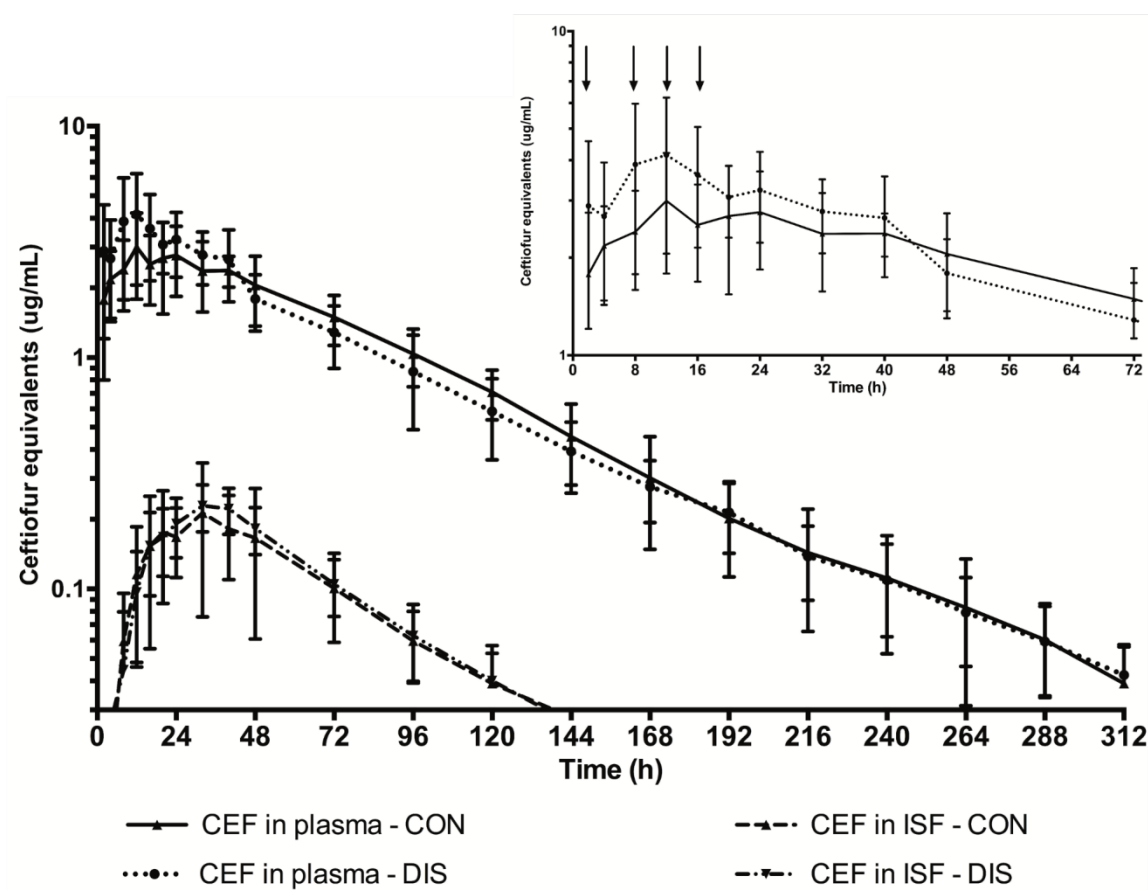


Figure 2. Semi-logarithmic transformations of mean plasma and interstitial fluid ceftiofur equivalent concentrations (\pm SD) for ten healthy cows (CON) versus ten cows with induced coliform mastitis (DIS) following a single subcutaneous administration of ceftiofur crystalline free acid. The concentration at which the x-axis intersects the y-axis represents the level of quantification for the analytical assay. The insert represents the same data, except only for plasma during the first 72 hours. The arrows indicate the time points where there are significant differences between the mean plasma concentrations.

Table 1. Plasma pharmacokinetic parameters for ceftiofur for ten cows with induced coliform mastitis (DIS) compared to ten healthy cows (CON) following a single subcutaneous injection of 6.6 mg/kg of ceftiofur crystalline free acid at the base of the ear. Results are presented as arithmetic mean \pm SE.¹

Parameter	Disease (DIS)	Control (CON)
λ_z (h ⁻¹)	0.012 \pm 0.0007 (0.012)	0.015 \pm 0.0006 (0.015)**
T _{1/2 λ_z} (h)	58.52 \pm 4.0 (57.35)	45.87 \pm 2.08 (45.47)**
C _{max} (µg/mL)	4.74 \pm 0.58 (4.43)	3.47 \pm 0.35 (3.33)*
T _{max} (h)	15.0 \pm 2.62 (12.4)	15.4 \pm 2.39 (12.8)
AUC ₀₋₂₄ (µg/mL x h)	78.01 \pm 9.0 (73.29)	57.82 \pm 5.66 (55.44)*
AUC _{0-∞} (µg/mL x h)	263.3 \pm 13.3 (260.1)	253.03 \pm 11.91 (251.2)
V _z /F (L/kg)	2.135 \pm 0.134 (2.098)	1.745 \pm 0.090 (1.723)**
CL/F (mL/h/kg)	25.69 \pm 1.42 (25.36)	26.45 \pm 1.03 (26.27)
MRT _{0-∞} (h)	66.87 \pm 4.23 (65.68)	72.14 \pm 4.57 (70.87)
F (%)	98.8 \pm 0.25 (98.8)	110.0 \pm 0.03 (110.0)**

¹Values in parentheses are the geometric mean.

**Means within the columns differ (P < 0.05).

*Means within the columns differ (P < 0.10).

λ_z (1/h), slope of the terminal phase; T_{1/2 λ_z} (h), terminal half-life; C_{max} (µg/mL), maximum plasma concentration; T_{max} (h), time of C_{max}; AUC_{0-24hr} (µg/mL x h), area under the curve from T0 to T24 hours; AUC_{0-∞} (µg/mL x h), area under the curve extrapolated to infinity using the equation $\frac{C_{last}}{\lambda_z}$; V_z/F (mL/kg), volume of distribution per fraction of the dose absorbed; and CL/F (mL/h/kg), clearance per fraction of the dose absorbed; MRT_{0-∞} (h), mean residence time extrapolated to infinity using the equation $\frac{C_{last}}{\lambda_z}$; and F, apparent bioavailability.

Table 2. Comparison of the elimination rate constant (λ_z) of CEF determined via the intravenous route and the absorption rate constant (k_a) of CE determined following administration of CEF CFA via the subcutaneous route.

Cow	Elimination rate constant (λ_z)	Absorption rate constant (k_a)	Ratio k_a/λ_z
Control group (CON)			
9156	0.085	0.019	0.22
9244	0.055	0.015	0.27
9325	0.113	0.015	0.14
9353	0.114	0.016	0.14
9480	0.110	0.014	0.12
9657	0.110	0.013	0.12
9725	0.109	0.011	0.10
9760	0.130	0.017	0.13
Mean (CON)	0.100	0.015	0.16**
Disease group (DIS)			
8972	0.081	0.018	0.22
9146	0.089	0.017	0.19
9206	0.045	0.018	0.39
9233	0.067	0.020	0.30
9389	0.082	0.020	0.24
9456	0.056	0.013	0.23
9709	0.103	0.027	0.26
9728	0.062	0.016	0.27
9776	0.077	0.018	0.24
9779	0.065	0.012	0.18
Mean (DIS)	0.070	0.018	0.25**

**Means within the columns differ ($P < 0.05$).

Table 3. Comparative interstitial fluid pharmacokinetic parameters (arithmetic \pm SE¹) for ceftiofur in DIS and CON cows (n=10) following a single injection of 6.6 mg/kg of ceftiofur crystalline free acid.

Parameter	Disease (DIS)	Control (CON)
λ_z (h ⁻¹)	0.018 \pm 0.0012 (0.018)	0.020 \pm 0.0012 (0.020)
T _{1/2 λ_z} (h)	39.75 \pm 3.03 (39.02)	35.06 \pm 2.16 (34.54)
C _{max} (µg/mL)	0.24 \pm 0.047 (0.21)	0.24 \pm 0.015 (0.24)
T _{max} (h)	33.2 \pm 2.53 (32.3)	35.2 \pm 1.77 (34.7)
AUC ₀₋₂₄ (µg/mL x h)	2.75 \pm 0.49 (2.48)	2.30 \pm 0.27 (2.15)
AUC _{0-∞} (µg/mL x h)	15.72 \pm 1.56 (15.26)	16.17 \pm 1.33 (15.74)
MRT _{0-∞} (h)	69.84 \pm 3.51 (69.24)	65.54 \pm 2.51 (65.16)

¹Values in parentheses are the geometric mean.

λ_z (1/h), slope of the terminal phase; T_{1/2 λ_z} (h), terminal half-life; C_{max} (µg/mL), maximum ISF concentration; T_{max} (h), time of C_{max}; AUC_{0-24hr} (µg/mL x h), area under the curve from T0 to T24 hours; AUC_{0-∞} (µg/mL x h), area under the curve extrapolated to infinity using the equation $\frac{C_{last}}{\lambda_z}$; CL/F (mL/h/kg), and MRT_{0-∞} (h), mean residence time extrapolated to infinity using the equation $\frac{C_{last}}{\lambda_z}$.

Table 4. Kidney concentration of desfuoylceftiofur cysteine disulfide (DCCD) and calculated desfuoylceftiofur (DFC) in DIS cows (n=10) following a single SQ injection of 6.6 mg/kg of ceftiofur crystalline free acid.

Cow	Measured DCCD concentration (µg/g)	Calculated DFC concentration ¹ (µg/g)
8972	<LOD	-
9146	<LOD	-
9206	0.094	0.38
9233	<LOD	-
9389	0.057	0.32
9456	<LOD	-
9709	<LOD	-
9728	<LOD	-
9776	<LOD	-
9779	<LOD	-

<LOD = below the limit of detection of the assay

¹ Desfuoylceftiofur (DFC) concentrations were calculated from DCCD values.

CHAPTER 5

EFFECT OF CEFTIOFUR TREATMENT ON ANTIMICROBIAL RESISTANCE AND *BLA*_{CTX-M} AND *BLA*_{CMY} RESISTANCE GENES IN DAIRY CATTLE WITH CLINICAL MASTITIS VERSUS HEALTHY CATTLE

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Abstract

There are concerns that the use of broad spectrum antimicrobials in veterinary medicine may lead to the development of antimicrobial resistance in animals and that these resistance genes may be transmitted to humans. Previous research has indicated that ceftiofur (CEF) treatment results in a transient change in resistance amongst fecal bacterial populations. Resistance amongst Enterobacteriaceae is commonly associated with the *bla*_{CMY-2} or *bla*_{CTX-M} gene. The objectives of this study were: 1) to isolate bacteria in the blood of cows afflicted with moderate or severe clinical mastitis and determine AMR patterns of these isolates; 2) to enumerate total fecal *E. coli* bacteria and resistant *E. coli* to CEF following administration of CEF CFA versus non treated control animals; 3) to compare phenotypic antimicrobial resistance and underlying genetic mechanisms from isolated bacteria; and 4) to determine if there was an impact of clinical disease on the development of AMR. The hypothesis was that administration of CEF would result in altered resistance patterns in diseased animals compared to healthy animals. Coliform blood culture prevalence was 9.1%. Following CEF treatment, there was a decrease in total *E. coli* cfu count and an increase in CEF resistant *E. coli* and proportion of CEF resistant *E. coli*. There was a significant effect of treatment ($P=0.012$) on resistant *E. coli* and a treatment ($P=0.0191$) and day ($P=0.011$) effect on the proportion of resistant bacteria. All CEF resistant isolates were also resistant to ceftriaxone and ampicillin. In addition, 64.3%

were also resistant to tetracycline. Forty-eight of 129 CEF resistant isolates harbored the *bla*_{CTX-M} gene, while nine harbored the *bla*_{CMY} gene, and two isolates harbored both genes. Isolates that were determined to be phenotypically CEF resistant were resistant to 4.72 while isolates that were CEF sensitive were resistant to 1.1 antimicrobials. The current study suggests that changes in bacterial populations following clinical disease are not different from those of healthy cows treated with CEF.

Key words: ceftiofur crystalline free acid, antimicrobial resistance, *bla*_{CTX-M}, *bla*_{CMY}, dairy cattle

Introduction

Ceftiofur (CEF) is a commonly used third-generation cephalosporin antimicrobial in the dairy industry (Zwald et al., 2004; Swant et al., 2005; Schuler et al., 2017). There are concerns that the use of broad spectrum antimicrobials in veterinary medicine may lead to the development of antimicrobial resistance (AMR) in animals and that these resistance genes may be transmitted to humans (WHO, 2017), resulting in AMR against antimicrobials like ceftriaxone. Ceftriaxone is also a third-generation cephalosporin often used in human medicine for the treatment of invasive enteric salmonellosis in children (Whichard et al., 2005; Tragesser et al., 2006). Currently, extended spectrum beta-lactamases (ESBL) are the primary cause of resistance within human Enterobacteriaceae isolates on a worldwide basis (Zhao and Hu, 2013). This has led to the World Health Organization to classify 3rd, 4th, and 5th generation cephalosporins amongst the highest priority critically important antimicrobials for the preservation of human health (WHO, 2017).

Escherichia coli is a common mastitis pathogen (Oliveira et al., 2013; NMC, 1999), which is primarily of environmental origin (NMC, 1999). In 2001, Wenz et al. estimated the prevalence of bacteremia secondary to moderate and severe clinical mastitis to be 32% (Wenz et al., 2001a). For this reason, many veterinarians include systemic antimicrobials, like CEF, in clinical mastitis treatment protocols in order to reduce the potential negative effects of a bacteremia (Schuler et al., 2017). In the manuscript describing the prevalence of bacteremia, the authors suggested that the source of these bacteria was likely enteric (Wenz et al., 2001a).

In a survey of 18 OH dairy herds, Tragesser et al. (2006) collected 1,266 fecal samples from 18 dairy herds to determine the level of ceftriaxone resistance and attempted to correlate this resistance with ceftiofur use. They found that 34.4% of the *E. coli* isolates had resistance to ceftriaxone and at least one cow exhibited ceftriaxone resistance in 67% of the surveyed herds. In herds that reported usage of CEF within the previous 6 months, the mean ceftriaxone resistance was 40% (range 0-97%), while the mean resistance in herds that reported no ceftiofur usage was 9% (range 0-34%). The presence of the *bla*_{CMY-2} gene was found in 83% of the resistant isolates. The research group determined that there was a herd level association between the use of CEF and ceftriaxone resistance, but not a cow level association.

The next year, Lowrance et al. (2007) published similar results following the treatment of beef feedlot steers with various dosage regimens of CEF CFA compared to commingled, non-treated control animals. In this work, they found 68.4% of fecal *E. coli* isolates harbored phenotypic resistance to at least one antimicrobial. Following CEF therapy, treated animals experienced transient increases in CEF resistant *E. coli*

populations, which returned to pre-treatment levels in approximately 2 weeks. As with the Tragessor et al. (2006) work, this research group determined a high level of correlation with CEF resistance and co-resistance against ampicillin, chloramphenicol, streptomycin, sulfisoxazole, and tetracycline (ACSSuT phenotype). Additionally, they described an association between CEF usage and reduced susceptibility to ceftriaxone. While no direct testing of genetic determinants of resistance were undertaken in this trial, the authors hypothesized that resistance to CEF and ceftriaxone was likely due to the *bla*_{CMY} gene.

In the US, third generation cephalosporin resistance in *E. coli* and *Salmonella enterica* isolates from food animals from 2000 – 2010 was most commonly associated with the plasmid borne *bla*_{CMY-2} gene (Daniels et al., 2009; Davis et al., 2015). However, in 2010, Wittum et al. reported the first dairy cattle isolation of *E. coli* with *bla*_{CTX-M} from sick and healthy dairy cattle in OH. CTX-M β -lactamase enzymes were first described in 1986 from a canine fecal *E. coli* isolate and have since been described worldwide (Bonnet, 2004). CTX-M β -lactamase enzymes typically result in high levels of resistance in *E. coli* against ampicillin, amoxicillin, carbenicillin, ticarcillin, piperacillin, cephalothin, cephaloridine, and cefuroxime, but maintain susceptibility to ceftiofur, β -lactamase inhibitors, and carbapenems (Bonnet, 2004; Wittum et al., 2010). These genes are predominantly responsible for the ESBL phenotype in *E. coli* and *Klebsiella* spp. in humans worldwide (Bonnet, 2004; Zhao and Hu, 2013).

The concern with CEF usage in food animals is that fecal contamination of meat products may result in the transfer of resistance genes to humans (US FDA, 2016b). However, as shown in the studies cited previously, presence of AMR in NTS fecal *E. coli* seems to be transient following CEF therapy. To reduce the prevalence of AMR genes in

feces of processed livestock, computer models utilizing pharmacological, microbiological, and animal production components have been developed to predict a post-treatment resistance reversion period. These would be similar to withdrawal times to reduce the risk of violative drug residues being present at processing (Volkova et al., 2016). However, there is little to no literature as to whether disease has any impact on the transient nature of AMR in NTS fecal *E. coli*.

The objectives of this study were: 1) to isolate bacteria in the blood of cows afflicted with moderate or severe clinical mastitis and determine AMR patterns of these isolates; 2) to enumerate total fecal *E. coli* bacteria and CEF resistant *E. coli* following administration of CEF CFA versus non-treated control animals; 3) to compare phenotypic antimicrobial resistance and underlying genetic mechanisms from isolated bacteria; and 4) to determine if there was an impact of clinical disease, like mastitis, on the development of AMR. The hypothesis was that administration of CEF would result in altered resistance patterns in diseased animals compared to healthy animals.

Materials and Methods

Animals and Eligibility Criteria

This study was completed at the Iowa State University Dairy Farm. The lactating herd consists of approximately 400 animals (approximately 90% Holstein and 10% Jersey), with 365-day rolling herd averages per cow of 10,991 kg milk, 404 kg fat, and 342 kg protein. Cows were eligible for the trial if they had not been treated with systemic or intramammary CEF within the past 20 days and were healthy prior to enrollment. Furthermore, the cows were thirty or more days from their next scheduled dry period.

Cows were housed in a free-stall barn bedded with recycled manure solids, which is standard practice for this dairy. Cows were fed a total mixed ration and watered, *ad libitum*. Ration parameters met or exceeded those recommended by the NRC guidelines (NRC, 2001). Cow housing and management met or exceeded the recommendations listed in the *Guide for Care and Use of Agricultural Animals in Research and Teaching* (FASS, 2010). Cows were milked by farm personnel three times each day (4 am, 12 pm, and 8 pm). Iowa State University's Institutional Animal Use and Care Committee approved the research protocol prior to commencement of trial procedures (protocol number 6-15-8030-B).

Experimental design – Detection of bacteremia

Cows detected with clinical mastitis by farm personnel were referred to the trial veterinarians for a full physical exam, including assignment of a severity score, as previously described by Wenz et al. (2001b). An aseptically collected milk sample was collected for bacterial culture. Cows identified as having either moderate or severe clinical mastitis that met the above selection criteria were enrolled in the trial.

Prior to administration of any medication, cows were restrained with a halter to expose one jugular vein to facilitate blood collection. The area over the jugular vein was clipped and then disinfected using at least three alternating scrubs of chlorhexidine and 70% ethanol. Following skin prep, 10 mL of blood was collected into a sterile 12-mL syringe via an 18-gauge x 1-inch needle. A new needle was placed on the syringe and the blood was aseptically transferred to a blood culture vial (BD Bactec, Fisher Scientific, Waltham, MA) containing sodium polyanethol sulfate, which binds β -lactam

antimicrobials that may be present in the blood. A second blood sample was collected for blood culture 48 hours later using the same technique as described above.

Following the first blood collection, cows received CEF hydrochloride (Excenel, Zoetis Inc., Kalamazoo, MI) at a dose of 2.2 mg/kg via the IM route. In addition, cows were allowed to receive any ancillary therapies prescribed in the farm's veterinary treatment protocols, at the discretion of the trial veterinarian assessing the animal. Ceftiofur therapy was continued for a total of 3 or 5 days and ancillary therapies provided at the discretion of the examining veterinarian, following the daily examination of the cow per the trial protocol.

Microbiological analysis of blood culture vials

Inoculated vials were transferred to the laboratory where a vent was aseptically placed in the vial per the manufacturer's instructions and then incubated at 37 °C. Blood culture vials were sub-cultured onto 5% sheep blood agar and MacConkey agar (Remel Microbiology Products, Lenexa, KS) 1, 2, and 7 days following inoculation by dripping 2-3 drops of solution onto each agar plate. Plates were then be streaked for isolation and incubated at 37 °C. Plates were read 24 and 48 hours after streaking. Bacteria isolated on culture plates were identified using standard techniques and confirmed with MALDI-TOF (MALDI Biotyper, Bruker Daltonics Inc., Billerica, MA). After completion of bacterial identification, isolates were frozen in a 50:50 mixture of brain heart infusion media and glycerol at -80 °C for later testing.

Microbiological analysis of milk samples

The milk sample was transported to the laboratory and cultured on 5% sheep blood agar and MacConkey agar plates (Remel Microbiology Products, Lenexa, KS) as described by the National Mastitis Council (NMC, 1999). The plates were examined the following day and confirmed as a coliform based on growth on MacConkey agar and typical colony morphology.

Experimental design – Enumeration of fecal *E. coli* organisms

Thirty Holstein cows were utilized in three separate groups to complete the objectives of the trial. The treatment group (TRT) consisted of cows that developed moderate or severe clinical mastitis (CM) using a scoring system described by Wenz et al. (2001b). Subsequently, two cows were selected from a pool of available cows that were within the same lactation group and approximately the same days in milk. One of those cows was assigned as the positive control (PC) and the other the negative control (NC). An attempt was made to select cows from the same pen and maintain those cows in this pen throughout the remainder of the trial, but this did not occur with all groups due to issues with management of cow flow. Cows were eligible for the trial using the same inclusion criteria as described above.

Following identification of the case of CM, the cow had a milk sample collected from the mastitic quarter for culture following aseptic preparation of the teat. The milk sample was cultured as described previously. Additionally, a fecal sample was collected from the rectal vault using a clean obstetrical sleeve. The fecal sample was transferred to a

50-mL conical tube, marked as Day 0 (D0), placed on ice, and transported to the laboratory for bacterial enumeration.

The cow was then weighed and treated with a single dose of CEF CFA (Excede, Zoetis Inc., Kalamazoo, MI) at 6.6 mg CEF equivalents/kg of body weight, administered at the base of either ear following instructions on the package insert. As part of the trial design, additional ancillary therapies (anti-inflammatories, fluid support, etc.) were administered per the farm's treatment protocols as determined by veterinarians on the research team. Additional antimicrobial therapies were not administered throughout the remainder of the trial. If the TRT cow was severely affected and showing clinical signs of muscular weakness, she was moved to the hospital pen until an improvement in clinical illness occurred. Otherwise she remained in her home pen throughout the trial. Cows in the PC and NC group were not enrolled until the next day, when results of the milk culture confirmed the causative agent was a coliform.

Upon confirmation of the culture result, fecal samples were collected from the PC and NC cows and processed in the same manner as the TRT cow. Cows in the PC group were then weighed and treated as described previously. Cows in the PC and NC groups were then returned to their home pen. All groups had additional fecal samples collected and processed in a similar manner as described above on days 3, 7, 14, 21, and 28 days following enrollment.

Microbiological analysis of fecal samples

Enumeration of NTS *E. coli* and CEF resistant *E. coli* was completed using a direct plating method on plain MacConkey agar plates and MacConkey agar plates impregnated

with 8 µg/mL of CEF, which is equivalent to the minimum inhibitory concentration to determine *E. coli* resistance to CEF (CLSI, 2015). A 1 g aliquot of feces was placed into a sterile stomacher bag, diluted with 9 mL of peptone water, and placed in a stomacher device for 1 minute. Ten-fold serial dilutions were then completed in peptone water from the bag up to 10^{-5} . From each of the dilutions, 100 µL was pipetted onto duplicate plain MacConkey plates and duplicate MacConkey plates with CEF. Plates were incubated for 18-24 hours at 37 °C. Following incubation, total NTS *E. coli* and CEF resistant *E. coli* were enumerated based on colony morphology. Colony counts from duplicate plates were averaged to determine the cfu count at each time point.

Up to three CEF resistant *E. coli* colonies were individually isolated onto trypticase soy agar plates and incubated for 24 hours at 37 °C for further screening. Confirmation of *E. coli* identification was completed using a spot indole test. Further identification steps were not completed as the combination of growth on selective media, colony morphology, and results of the spot indole test provided >99% probability of correct identification (Kanwar et al., 2013). Isolates were then transferred to a 50:50 mixture of brain heart infusion broth and glycerol and frozen at -80 °C for later analysis.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was completed on all suspect CEF resistant *E. coli* fecal isolates and all *E. coli* isolates from blood culturing, post-mortem, and mastitis samples. Broth micro-dilution susceptibility analysis was completed using the gram-negative National Antimicrobial Resistance Monitoring System (NARMS) plate (CMV3AGNF, Thermo Scientific Trek Diagnostic Systems, Waltham, MA). On the day

prior to susceptibility testing, frozen isolates were struck onto 5% sheep blood agar plates and incubated overnight at 37 °C. To inoculate the susceptibility plates, 2-3 isolated NTS *E. coli* colonies were transferred to 5 mL of sterile 0.85% physiologic saline adjusted to a 0.5 McFarland standard using automated methods (Sensititre Nephelometer, Thermo Scientific Trek Diagnostic Systems, Waltham, MA). 10 µL of the adjusted standard was then transferred to 5 mL of Mueller-Hinton broth. Following vortexing, each well of the antimicrobial susceptibility plate was inoculated with 50 µL of broth suspension using an automated inoculator (Sensititre AutoInoculator, Thermo Scientific Trek Diagnostic Systems, Waltham, MA). Plates were then incubated for 18 hours at 37 °C and results recorded with an automated system (Sensititre ARIS 2X, Thermo Scientific Trek Diagnostic Systems, Waltham, MA). Determination of sensitive, intermediate, or resistant classification was based on the current version of the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2017) when available. As a breakpoint is not established azithromycin, a breakpoint of ≥ 32 µg/ml was used, as defined by NARMS (US FDA, 2016b). For data analysis, all isolates that were classified as intermediate were considered susceptible when converting to binary classification. Quality control of the antimicrobial susceptibility testing was completed using *E. coli* ATCC 25922 (American Type Culture Collection, Manassas, VA) and compared to quality control ranges recommended by CLSI (CLSI, 2015).

Following completion of antimicrobial resistance testing, enumeration of CEF resistant bacterial numbers were corrected to account for isolates that failed to confirm as resistant.

Determination of resistance genes

Determination of the presence of *bla*_{CTX-M} and *bla*_{CMY} resistance genes was completed using previously described PCR protocols (Zhao et al., 2001; Zhao and Hu, 2013; Davis et al., 2015). Briefly, boiled cell lysates were used for the reaction template. Standard amplification reaction volumes were 25-μL final concentrations of 1x PCR buffer, 200 μM of each deoxynucleoside triphosphate (dNTP), 1.5 μM MgCl₂, 1 pmol/μL primer, and 2.5 U Taq. Cycling conditions for *bla*_{CMY-2} PCR included an initial denaturation of 95 °C for 15 minutes, and 35 cycles of 94 °C for 30 seconds, 55 °C for 30 seconds, 72 °C for 1 minute, and 70 °C for 10 minutes. Cycling conditions for the *bla*_{CTX-M} PCR included an initial denaturation of 95 °C for 15 minutes, and 35 cycles of 94 °C for 30 seconds, 52 °C for 30 seconds, 72 °C for 1 minute, and 70 °C for 10 minutes. Primer sequences are shown in Table 1. Positive control samples were field isolates that were obtained from the Centers for Disease Control. Visualization and separation of PCR amplicons were performed using the QIAxcel Advanced capillary electrophoresis system (QIAGEN, Hilden, Germany). The PCR positive samples were purified and sequenced at The DNA Facility of the Iowa State University Office of Biotechnology using standard Sanger sequencing (3730xl DNA Analyzer, Applied Biosystems, Foster City, CA). Contig assembly was performed using the SeqManPro module in DNASTar (DNA Star Lasergene, Madison, WI). Comparisons among the sequences in this study were made using Clustal W alignment, and the phylogenetic tree was generated using the Neighbor Joining Tree method (MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0, Tamura et al., 2013).

Data analysis

Statistical analysis was performed using a commercially available software program (SAS 9.4, SAS Institute, Cary, NC). Log cfu bacterial counts and proportions between groups were analyzed via the GLIMMIX procedure using repeated measures analyses of variance (ANOVA), with animal being the subject of repeated measures. Fixed effects were treatment (TRT, PC, or NC), time, and the interaction between treatment and time. Statistical significance was established when $P < 0.05$.

Results

Detection of bacteremia

Over the course of 13 months, 33 cows had blood cultures following development of clinical mastitis. Two cows from the sampled group were culture positive for *E. coli* and an additional cow was culture positive with *Klebsiella* spp. (9.1%; 95% CI= -1.3-19.4). There were eight cows that were culture positive with *Bacillus* spp. or staphylococci, which were assumed to be contaminants associated with sampling. The two cows from which *E. coli* was isolated were also culture positive for *E. coli* from the milk culture. In addition, one of these two cows died as a result of the clinical case and underwent a full post-mortem examination at the ISU Veterinary Diagnostic Laboratory. Liver tissue from this cow was culture positive for *E. coli*. These five *E. coli* isolates were included in the antimicrobial susceptibility testing and PCR analysis. Additionally, *E. coli* isolates from four of the IMM infections from the clinical cases in the enumeration study were retained for future analysis.

Enumeration of fecal *E. coli* organisms

At enrollment, there was no statistical difference for any of the animal enrollment variables between the treatment groups. During the study, one of the TRT group cows died between 14 and 21 days of the study from complications associated with the clinical mastitis case. One additional cow was enrolled in place of this cow to assure ten complete groups. However, her fecal isolates were still included in the pool to be analyzed along with the replacement animal.

In total, 265 fecal isolates that were initially screened as CEF resistant using the CEF impregnated agar plates were isolated for further testing. Comparing the isolates from each cow at each time point following completion of antimicrobial susceptibility and PCR analysis, 222 unique isolates were identified. After completion of antimicrobial susceptibility testing, 129 of the 222 (58.1%, 95% CI=51.6-64.6) fecal isolates that were originally screened as resistant using CEF impregnated agar plates were confirmed to be phenotypically resistant.

Estimated log total *E. coli* counts are displayed in Figure 1. There was not an effect of treatment on total cfu count, but there was an impact by day ($P<0.0001$) and treatment by day interaction ($P=0.049$). At day 0, all of the cows had approximately the same total *E. coli* count. Cows that received CEF (TRT and PC) had a significant reduction in total cfu count from day 0 to 3 in the PC group ($P=0.0063$) and from day 0 to 7 in the TRT group ($P=0.0001$). From these time points, the total cfu count rebounded towards the pre-treatment levels. Throughout the entire time period, the NC group had no significant changes in cfu count.

Estimated log CEF resistant *E. coli* counts are displayed in Figure 2. There was a significant effect of treatment ($P=0.012$), but no effect by day or treatment by day interaction. Prior to treatment with CEF, the TRT cows had a numerically higher CEF resistant cfu count compared to the PC and NC groups. Following treatment, both the TRT and PC groups had an increase in cfu counts associated with resistant *E. coli* organisms. In the TRT group, peak CEF resistant organisms occurred on day 3 resulting in a statistically significant difference compared to the NC group ($P=0.0373$), which continued through day 7 ($P=0.379$). There was a tendency for the PC group to be significantly lower than the TRT group at day 7 ($P=0.0671$). By day 14, resistant bacteria in the TRT group returned to pre-treatment levels and there was no difference in the three groups throughout the remainder of the monitoring period.

The proportion of total bacteria represented by resistant *E. coli* are shown in Figure 3. There was significant effect of treatment ($P=0.0191$) and day ($P=0.011$), but no effect of the treatment by day interaction. Similar to the resistant cfu count, the proportion increased from day 0 to day 3 for both the TRT and PC groups and then decreased until day 14, when they were approximately the same for all groups numerically. The TRT group was significantly different from the NC group at day 3 ($P=0.0436$) and day 7 ($P=0.0147$).

As stated above, from the fecal isolates, 265 were initially determined to be CEF resistant using the CEF impregnated plate method. All of these samples, plus ten samples from mastitis cases in the TRT group and from systemic cultures (blood cultures and post-mortem exams), underwent antimicrobial susceptibility testing. All plates had correct responses for individual positive and negative controls. All quality control samples met

CLSI recommended ranges, with the exception of tetracycline for which all outcomes were 4 µg/mL compared to the reference range of 0.5-2 µg/mL (CLSI, 2015).

From the 10 non-fecal origin samples, eight were pan-susceptible while one was resistant to tetracycline and another was resistant to ampicillin, streptomycin, sulfisoxazole, and tetracycline.

Antimicrobial susceptibility testing results for the 129 unique isolates that were CEF resistant are shown in Table 2. All 129 CEF resistant isolates were also resistant to ceftriaxone and ampicillin. In addition, 83 (64.3%; 95% CI=56-72.7%) of the CEF resistant isolates were also resistant to tetracycline. Including all 222 unique fecal isolates, 140 and 156 isolates were resistant to ceftriaxone and ampicillin, respectively.

In total, 55 fecal isolates had at least one β -lactamase resistance gene detected by PCR. Forty-eight harbored the *bla*_{CTX-M} gene while nine harbored the *bla*_{CMY} gene, with two of these isolates harboring both genes. A dendrogram of the phylogenetic tree for *bla*_{CTX-M} is shown in Figure 4, while the *bla*_{CMY} isolates are shown in Figure 5. Phylogenetic analysis indicated that the *bla*_{CTX-M} isolates were 98 to 100% similar, while the *bla*_{CMY} were 99.7-100% similar. Seventy-seven isolates had CEF resistance mechanisms other than *bla*_{CTX-M} or *bla*_{CMY}.

The distribution of days in the trial when an isolate was identified with *bla*_{CTX-M} is shown in Figure 6. Of the 48 *bla*_{CTX-M} positive isolates, 37 occurred on separate cow days. This indicates that there were 11 isolates for which two unique isolates were found from the same cow on the same day. Eighteen of 21 the cows that were treated with CEF had at least one point in time when an isolate was positive for *bla*_{CTX-M} versus only 4 of the cows in the NC group. The *bla*_{CTX-M} gene was more likely to be found on day 0 or 3 and by day

28, no cows had a *bla*_{CTX-M} isolate present. In contrast, the *bla*_{CMY} gene was equally distributed across the follow-up period (data not shown). This gene was found in isolates from five TRT cows and 3 NC group cows.

Histograms representing the total number of antimicrobials for which isolates are resistant are shown in Figure 7. Isolates that were determined to be phenotypically CEF resistant were resistant to 4.72 antimicrobials on average (range 3-8; 95% CI=4.53-4.91), while isolates that were CEF sensitive were resistant to 1.1 antimicrobials (range 0-7; 95% CI=0.69-1.5). Isolates that were *bla*_{CTX-M} positive were resistant to 4.7 antimicrobials (range 3-7; 95% CI=4.42-5.05), while those with the *bla*_{CMY} gene were resistant to 5.5 antimicrobials (range 5-8; 95% CI=4.61-6.4). Bacterial isolates that were resistant to CEF and did not contain either of the *bla* genes were resistant to 4.65 antimicrobials (range 3-8; 95% CI=4.11-4.89).

For isolates that were CEF resistant, resistance to four antimicrobials had the highest frequency (n=60). The resistance phenotype was ampicillin, CEF, ceftriaxone, and then either tetracycline (n=51) or streptomycin. This phenotype was also the most common amongst the *bla*_{CTX-M} positive isolates (n=17). If the phenotype was resistant to five antimicrobials (n=40), the most common phenotype present was amoxicillin/ clavulanic acid, ampicillin, cefoxitin, CEF, and ceftriaxone (n=29). The remaining 11 isolates resistant to five antimicrobials showed the ampicillin, CEF, ceftriaxone, streptomycin, and tetracycline phenotype (n=9). The other two showed the same phenotype, except one with resistance to cefoxitin and one to chloramphenicol instead of tetracycline.

Isolates carrying the *bla*_{CTX-M} gene that were resistant to six antimicrobials (n=12) always exhibited the ampicillin, CEF, ceftriaxone, chloramphenicol, streptomycin, and

tetracycline phenotype. Nine *bla*_{CTX-M} positive isolates that were resistant to five antimicrobials predominantly exhibited the ampicillin, CEF, ceftriaxone, streptomycin, and tetracycline phenotype. From the isolates that were *bla*_{CTX-M} positive, only one of the isolates showed resistance to amoxicillin/ clavulanic acid, but it also carried the *bla*_{CMY} gene.

All but one of the isolates carrying the *bla*_{CMY} gene were resistant to amoxicillin/ clavulanic acid. That isolate was also the only *bla*_{CMY} positive one that was not resistant to cefoxitin, which potentially brings the results of the antimicrobial susceptibility test into question for this isolate. The most common phenotype for *bla*_{CMY} positive isolates was amoxicillin/clavulanic acid, ampicillin, cefoxitin, CEF, and ceftriaxone.

If isolates had no resistance gene determined and were resistant to four antimicrobials, the resistance phenotype was ampicillin, ceftiofur, ceftriaxone, and tetracycline. In contrast, if the isolates were resistant to five antimicrobials and did not have a resistance gene present, the phenotype was amoxicillin/clavulanic acid, ampicillin, cefoxitin, ceftiofur, and ceftriaxone.

There were eight CEF-susceptible isolates that were resistant to amoxicillin/ clavulanic acid, ampicillin, cefoxitin, ceftriaxone, chloramphenicol, streptomycin, and tetracycline.

Discussion

In contrast to Wenz et al. (2001a), this study had a much lower prevalence of bacteremia. In the comparison study, a larger number of animals were sampled (n=144) over six farms. It is possible for there to be an influence of farm; however, risk by farm

was not reported in the comparison study. Additionally, the comparison study used a different blood culturing system than in the current trial due to discontinuation of the product (Wenz et al., 2001a).

In this study, bacterial isolates from mastitis cases and bacteremia isolation were more susceptible compared to fecal isolates. However, fecal isolates that underwent antimicrobial susceptibility testing were screened for CEF resistance and only selected based on the phenotype. Mastitis cases associated with *E. coli* are environmental in nature and would be expected to be associated with the fecal microbiota of the source dairy. Additionally, isolates from systemic samples were suggested to have an origin of the intestinal tract (Wenz et al., 2001a). The finding of a high level of susceptibility amongst *E. coli* mastitis isolates is consistent with recent submissions to diagnostic laboratories at Iowa State University (Adam Krull, DVM, personal communication) and the University of Minnesota (Erin Royster, DVM, personal communication).

Following CEF therapy, there was a reduction in total bacteria number and an increase in the number and proportion of CEF resistant *E. coli*. Following these initial changes, bacterial populations returned to pre-treatment levels by 14 days. This pattern was consistent with previous studies following CEF therapy (Jiang et al., 2006; Tragesser et al., 2006; Lowrance et al., 2007; Singer et al., 2008). To our knowledge, no one has investigated the impact of clinical mastitis on shedding patterns of CEF resistant bacteria. It appears from the current study that the pattern following clinical disease is not different than previous studies, despite the fact that the TRT group was numerically higher in CEF resistant cfu counts early in the observation period. Therefore, the hypothesis for this trial

was not proven. It is possible that this study did not have sufficient power to discern a true difference in resistant bacterial between the TRT and PC group.

All of the resistant CEF isolates were also resistant to ceftriaxone and ampicillin. However, there were eight isolates that were susceptible to CEF and resistant to ceftriaxone indicating different mechanisms may be present which drive ceftriaxone resistance compared to CEF.

It should be noted that the breakpoints utilized for determining susceptibility to fecal isolates were based on NARMS guidelines (US FDA, 2016b), which are primarily dictated by human antimicrobial susceptibility testing methodologies (CLSI, 2017). Therefore, susceptibility patterns determined in this study may not be relevant to bovine fecal isolates. Additionally, there are no antimicrobial susceptibility breakpoints established for bovine fecal *E. coli* isolates.

In total, 37% of the fecal isolates that were CEF resistant were carrying the *bla*_{CTX-M} gene. This is consistent with previous reports suggesting the *bla*_{CTX-M} gene is among the most prevalent reason for extended spectrum β -lactamase *E. coli* (Davis et al., 2015). From the 48 isolates, 37 were present on separate cow days, with 10 each of the 37 appearing on day 0 and day 3, respectively. By day 28, none of the fecal isolates were found to contain the gene. While there is no other research having reported this finding, it is prudent to not over-interpret the significance of this finding due to the relatively small population of bacterial isolates and the random nature in which isolates were originally selected for further analysis.

More than half of the CEF resistant isolates had resistance determinants that were not determined by the PCR analysis used in this study. In examining the phenotypic

resistance patterns of the *E. coli* isolates from this study, it is likely that several other resistance mechanisms were present in this microbial population. According to the NCBI website, there are 34 β -lactamase families currently recorded on three different websites (NCBI, 2017). In the future, it would be prudent to use a technique with higher resolution of the genome, such as whole genome sequencing. This approach would provide a more complete picture of the entire resistome of such a diverse bacterial population as the one studied here.

Previous research has suggested that *E. coli* isolates harboring the *bla*_{CMY} gene exhibited the ampicillin, chloramphenicol, streptomycin, sulfisoxazole, and tetracycline resistance phenotype (Tragessor et al., 2006; Lowrance et al., 2007). In the present work, none of the *bla*_{CMY} harboring isolates were resistant to sulfisoxazole. In addition, only three showed resistance to chloramphenicol. While it is not evident as to why this difference occurred, it is likely that a gene mutation occurred resulting in a narrower resistance phenotype in the current study.

A weakness of this study was not creating a NC group with no history of CEF usage at the individual animal level. That may have allowed for an assessment on the impact CEF therapy on resistance development. However, one of the goals was to mimic normal management of a commercial dairy farm in this trial. Additionally, the common use of CEF on the investigational farm and the potential for horizontal transmission likely limited the utility of such a control group (Boyer and Singer, 2012).

In conclusion, there was a correlation between CEF resistance and that of ceftriaxone and ampicillin. Initiation of CEF therapy resulted in a transient increase in resistant isolates, which returned to pretreatment levels in approximately 14 days.

However, this work did not show a difference in bacterial numbers or changes over time between healthy and diseased animals. Commonly described β -lactamase genes were prevalent in resistant isolates but apparently other resistance mechanisms are substantial contributors to the resistome. Future work is needed to more completely describe the full range of resistance mechanisms in order to better understand the complex interactions that occur amongst bacterial populations. In addition, these complex interactions limit the ability to discern the impacts to implementation of antimicrobial stewardship programs on the resistome without the use of higher resolution sequencing techniques.

Table 1. PCR primer sequences used for analysis of resistance genes. (Davis et al., 2015).

Gene	Primer name	Sequence (5' to 3')
<i>bla</i> _{CTX-M}	CTX-M.F	TTT GCG ATG TGC AGT ACC AGT AA
	CTX-M.R	CGA TAT CGT TGG TGG TGC CAT A
<i>bla</i> _{CMY}	CMY-F	GAC AGC CTC TTT CTC CAC A
	CMY-R	TGG AAC GAA GGC TAC GTA

Table 2. Distribution of mean resistance (95% CI) of 129 (ALL) *E. coli* isolates that were resistant to CEF as tested with the National Antimicrobial Resistance Monitoring System (NARMS) gram-negative plate compared to the three comparison groups (TRT, n=46; PC, n=33; & NC, n=28) following CEF therapy.

Antimicrobial	All	TRT	PC	NC
Amoxicillin/ Clavulanic Acid	27.2 (19.3-34.9)	29.8 (17.7-42.1)	25.6 (11.3-40)	24.2 (8.8-39.7)
Ampicillin	100	100	100	100
Azithromycin	0	0	0	0
Cefoxitin	27.9 (20-35.8)	31.6 (19.1-44)	25.6 (11.3-40)	24.2 (8.8-39.7)
Ceftiofur	100	100	100	100
Ceftriaxone	100	100	100	100
Chloramphenicol	17.0 (10.5-23.6)	19.3 (8.7-29.9)	20.5 (7.2-33.8)	9.1 (-1.2-19.4)
Ciprofloxacin	1.6 (-0.6-3.7)	0	2.6 (-2.6-7.7)	3 (-3.1-9.2)
Gentamicin	1.6 (-0.6-3.7)	0	2.6 (-2.6-7.7)	3 (-3.1-9.2)
Nalidixic Acid	1.6 (-0.6-3.7)	0	2.6 (-2.6-7.7)	3 (-3.1-9.2)
Streptomycin	32.6 (24.4-40.8)	36.8 (23.9-49.8)	33.3 (17.8-48.8)	24.2 (8.8-39.7)
Sulfisoxazole	0	0	0	0
Tetracycline	64.3 (56-72.7)	54.4 (41-67.7)	71.8 (57-86.6)	72.7 (56.7-88.8)
Trimethoprim/ Sulfamethoxazole	0	0	0	0

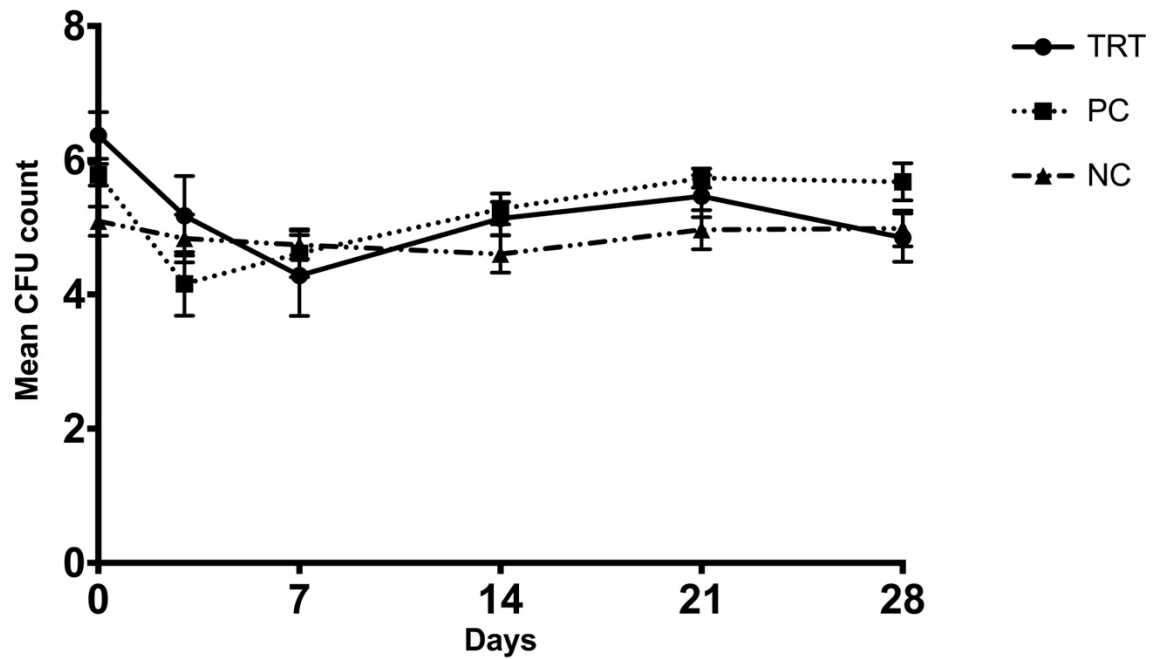


Figure 1. Log transformations of mean (± 1 SE) total fecal *E. coli* cfu with moderate or severe mastitis (TRT; n=10) and without disease (PC; n=10) following a single administration of ceftiofur crystalline free acid at 6.6 mg ceftiofur equivalents (CE)/kg of body weight versus healthy control cows (NC) that did not receive antimicrobial therapy (n=10).

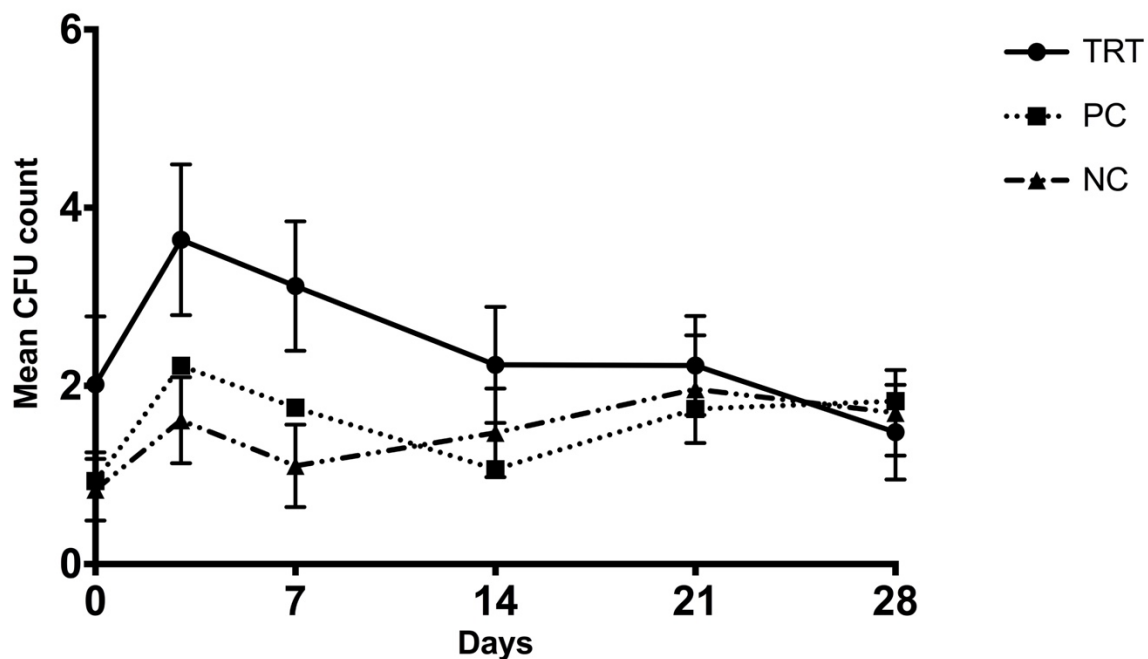


Figure 2. Log transformations of mean (± 1 SE) ceftiofur resistant fecal *E. coli* cfu from cows with moderate or severe mastitis (TRT; n=10) and without disease (PC; n=10) following a single administration of ceftiofur crystalline free acid at 6.6 mg ceftiofur equivalents (CE)/kg of body weight versus healthy control cows (NC; n=10) that did not receive antimicrobial therapy.

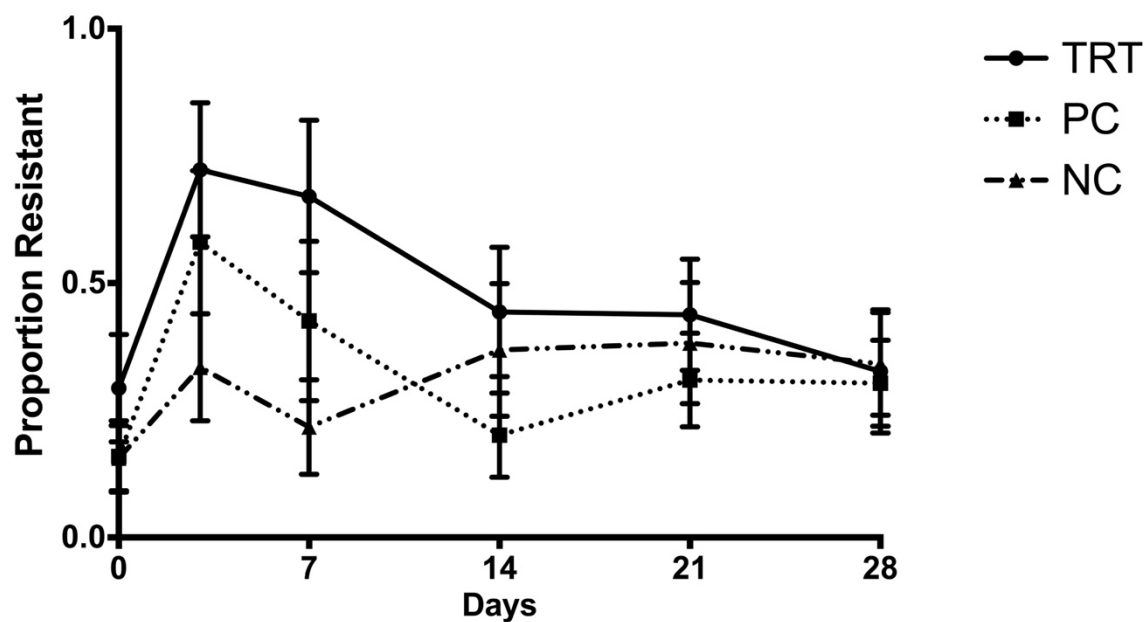


Figure 3. Proportion of log transformed ceftiofur resistant fecal *E. coli* cfu (± 1 SE) of total non-type specific *E. coli* from cows with moderated or severe mastitis (TRT; n=10) and without disease (PC; n=10) following a single administration of ceftiofur crystalline free acid at 6.6 mg ceftiofur equivalents (CE)/kg of body weight versus healthy control cows (NC; n=10) that did not receive antimicrobial therapy.

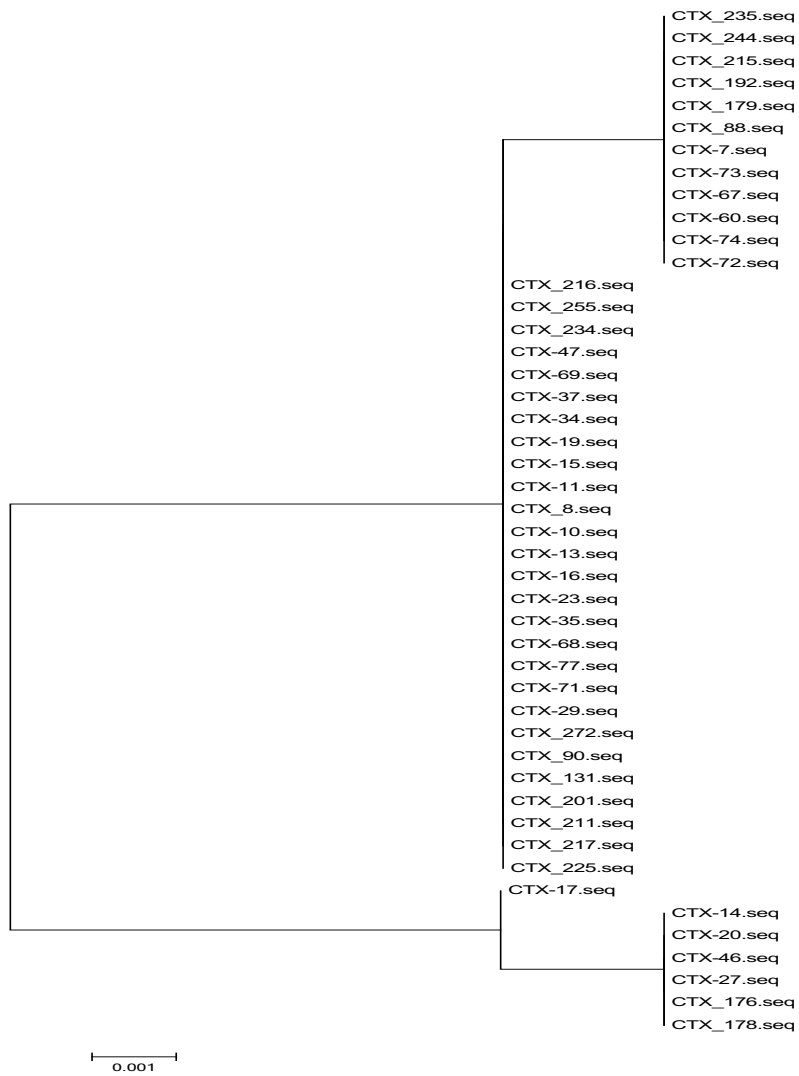


Figure 4. Dendrogram demonstrating the phylogenetic tree of the *bla*_{CTX-M} isolates.

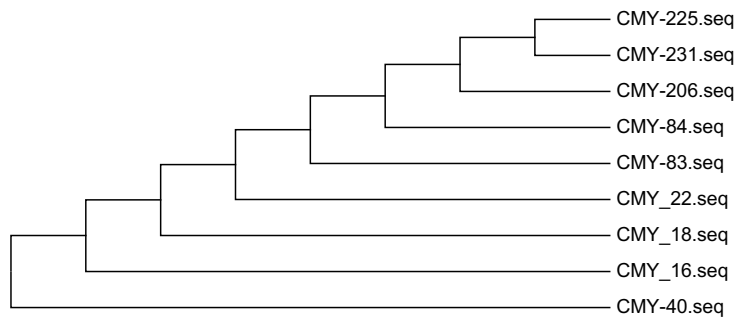


Figure 5. Dendrogram demonstrating the phylogenetic tree of the *bla*_{CMY} isolates.

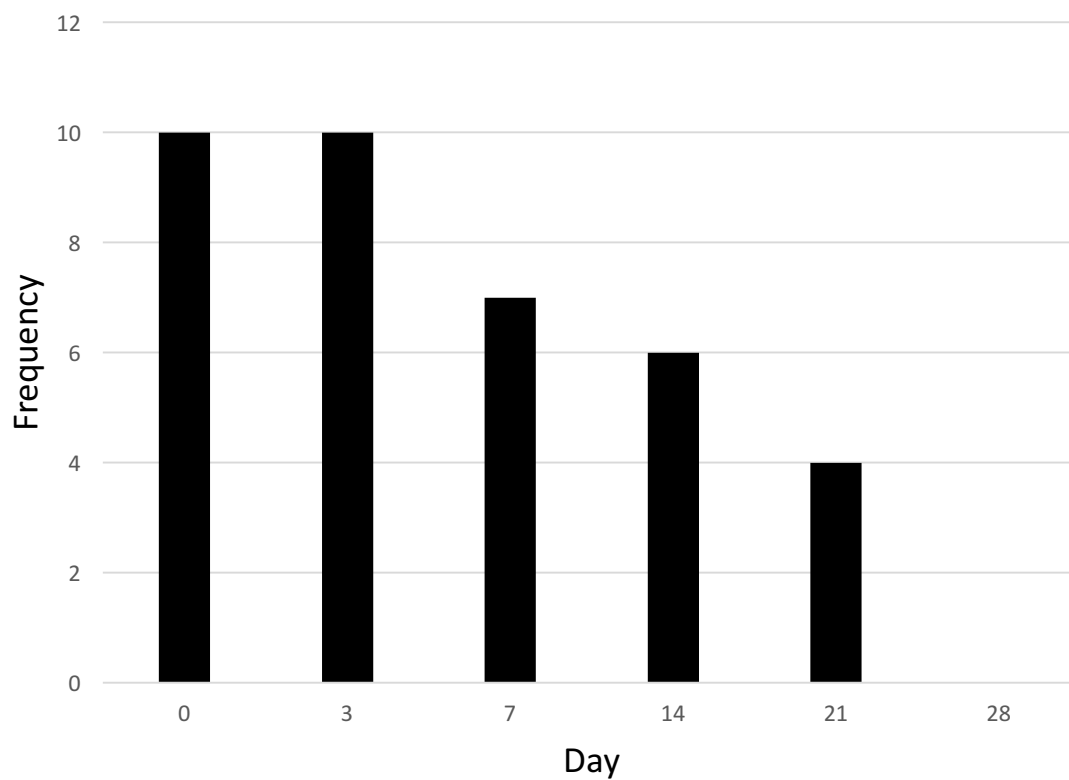


Figure 6. Frequency distribution of when *bla*_{CTX-M} was isolated.

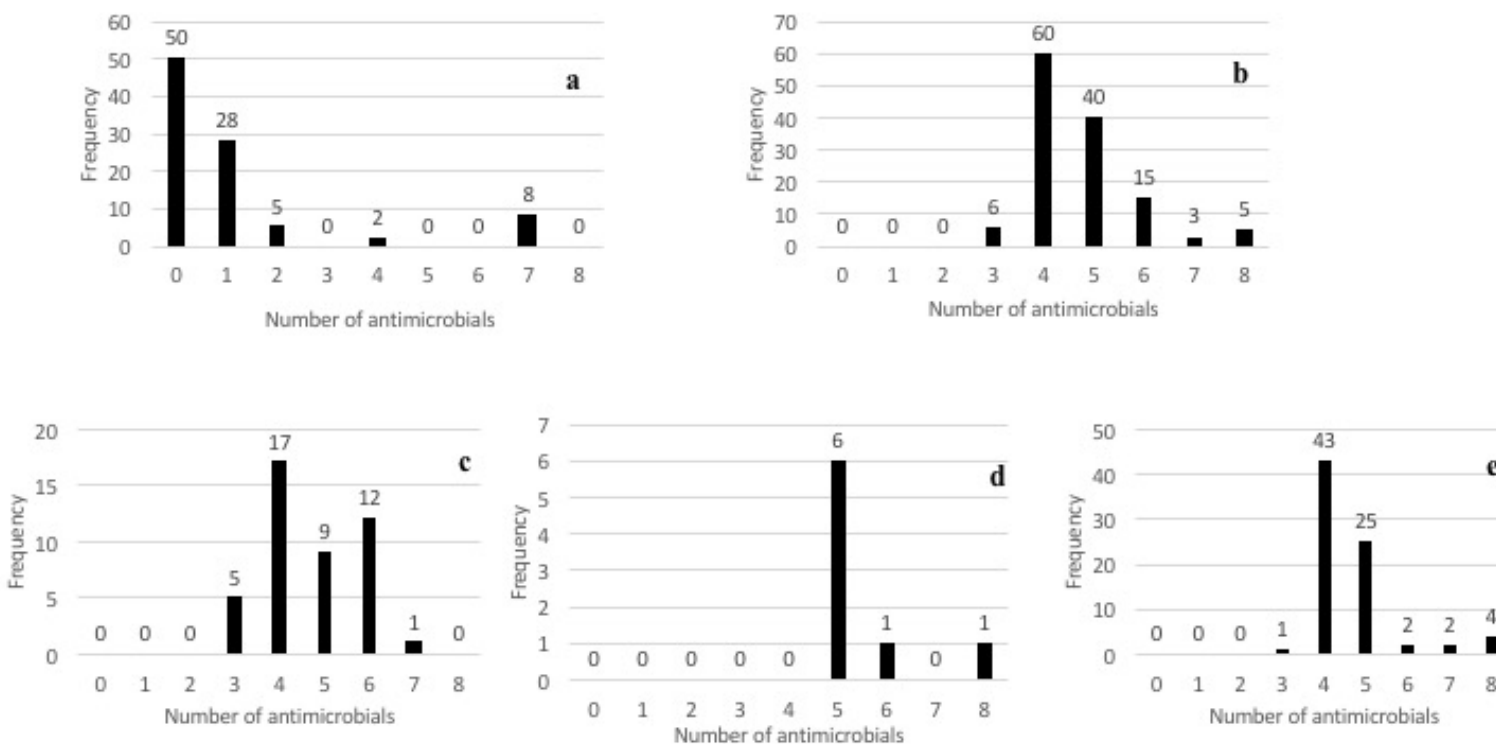


Figure 7. Frequency distribution of the number of antimicrobials that are resistant against. a) Isolates that were CEF susceptible; b) Isolates that were CEF resistant; c) Isolates determined to be carrying the *bla*_{CTX-M} gene; d) Isolates determined to be carrying the *bla*_{CMY} gene; and e) Isolates that were CEF resistant where neither gene was found.

CHAPTER 6. SUMMARY AND CONCLUSIONS

Since its introduction into the veterinary industry, the various formulations of ceftiofur (CEF) have been widely used in dairy cattle. While national estimates of CEF use are not available down to the animal level, a recent study indicates it is used nearly twice as often as other competitive products (Schuler et al., 2017). This is likely due its broad spectrum of activity and short duration of milk and meat withdrawals. However, despite its short withdrawal times, violative meat residues in cull dairy cattle are most commonly associated with CEF. Over the last ten years, CEF violative residues have gone from zero to the over 200 per year, surpassing penicillin as the most common residue in 2012 (Chapter 1, Figure 2). The potential reasons for the increase in violative residues include: marketing of animals before the meat withdrawal periods have elapsed; errors in record keeping regarding treatment status; changes in the tolerance and consequently the meat withdrawal intervals over the years; and potentially differences in animal health status compared to the status of the animals used during the drug approval process. In the drug approval process, healthy animals are utilized, whereas the majority of these drugs are not intended for and are not administered to healthy animals in clinical dairy practice. As a result of disease, there are potentially differences in drug absorption, distribution, metabolism, and excretion. The first objective of this dissertation was to study CEF pharmacokinetics (PK) in healthy versus diseased cattle and to investigate CEF tissue depletion following induction of coliform mastitis.

Two studies were conducted to investigate the PK of ill versus healthy animals. In the first study, CEF hydrochloride was administered over a five-day period to cows with naturally occurring coliform mastitis (Chapter 2) and in the second study, a single dose of CEF

crystalline free acid (CFA) was administered to cows with induced coliform mastitis (Chapter 4). In both of these studies, we were able to prove that certain PK parameters were significantly altered compared to healthy control cows. In the first study (Chapter 2), diseased cows had higher volume of distribution per fraction of the dose absorbed (V_z/F) and clearance per fraction of the dose absorbed (CL/F) compared to the healthy herdmates. The diseased group also had significantly lower steady-state maximum concentration ($C_{\max ss}$) and area under the concentration curve in the interval between drug doses (AUC_{τ}). Despite changes in V_z/F and CL/F , there was not a significant change in the elimination half-life ($T_{1/2 \text{ last dose}}$). In this study, the mean $T_{1/2 \text{ last dose}}$ in the control group was 35.8 hours, while in the mastitic group it was 41.9 hours. However, one of the cows had an elimination half-life of nearly two times the mean of the control group at 70.9 hours.

In a separate study (Chapter 4), cows were inoculated with IMM *E. coli* to induce the disease process. As a result, it was possible to again demonstrate significantly higher V_z/F in the diseased group but no differences in CL/F . Additionally, the diseased group had a significantly higher elimination half-life ($T_{1/2 \lambda z}$). Again, the cow with the longest $T_{1/2 \lambda z}$ was nearly twice as long as the mean of the control group. An elimination half-life that is twice as long indicates that the time to reach a regulatory tolerance level will take twice as long, provided the drug is not bound within the organ of elimination like may be the case with aminoglycosides (Riviere et al., 1998). At the conclusion of this study, cows were sacrificed and kidney tissues were analyzed for CEF residue concentrations. While none of the cows had residue levels above the tolerance for desfuroylceftiofur, the target metabolite for CEF, the cow with the longest $T_{1/2 \lambda z}$ had a kidney tissue concentration slightly below the tolerance. While this work did not prove that violative residues could occur as a consequence of utilizing

CEF in diseased cows, it should be noted that the cows in this latter study were not as clinically ill as the first study. Future work should focus on assessing tissue residues in cattle that are more severely affected by clinical disease in order to rule out the potential for creating violative residues when marketing these animals.

In the first study (Chapter 2), $C_{\max ss}$ and AUC_{τ} were significantly higher in the control group, leading to the question of whether a clinically effective concentration of CEF was reaching the bio-phase. Therefore, in the induced mastitis trial (Chapter 4), subcutaneous ultrafiltration probes were utilized to collect interstitial fluid and measure non-protein-bound CEF concentrations. In this study, there were no differences in ISF CEF PK between the two groups of cows. However, there also was no difference in plasma area under the curve extrapolated to infinity ($AUC_{0-\infty}$), even though there was a trend toward a higher C_{\max} and area under the curve in the first 24-hours after CEF dosing (AUC_{0-24}) in the disease group. A lack of difference in plasma PK would not be expected to result in differences in ISF PK.

In plasma, CEF is typically >90% protein-bound in the adult bovine (S. A. Brown et al., 1991). Drugs that are weak acids, like CEF, are primarily bound to albumin (Riviere, 2009a). In disease, albumin concentrations typically decrease to compensate for the liver's need to increase production of acute phase proteins (Ceciliani et al., 2012), which could lead to a higher unbound fraction of drugs that primarily bind to albumin. As CEF is a low extraction ratio drug, an increase in the unbound fraction will reduce the total concentration of the drug in the circulation, but not the free concentration (Toutain and Bousquet-Melou, 2002). This could have explained the lower AUC_{τ} seen in the first study (Chapter 2); however, we did not measure plasma albumin concentrations or the degree of protein binding, nor did we collect interstitial fluid to determine drug concentrations in that matrix. This would suggest that the

bio-phase CEF concentration will be unaffected by disease, if passive diffusion is the only mechanism involved in moving drug into the bio-phase. However, there may be other factors contributing such as efflux pumps (Foster et al., 2015) and pathological processes associated with clinical disease (Walker et al., 1994; Clarke et al., 1996; Washburn et al., 2005) that may contribute to different bio-phase concentrations depending on location in the body. Future PK studies investing the clinical efficacy of drugs need to involve measurement of drug concentrations within the bio-phase.

Ceftiofur sodium is reported to have nearly a 100% bioavailability (S. A. Brown et al., 1991) and the kinetics between CEF sodium and CEF hydrochloride are similar (Pharmacia and Upjohn, 1998). Additionally, bioavailability of CEF CFA has been reported to be 100% following IM administration in the equine (Collard et al. 2011). Bioavailability of CEF CFA has not been reported in the bovine to date. An objective of Chapter 4 of this dissertation was to describe the bioavailability of CEF CFA in healthy cattle versus cattle with induced coliform mastitis. The absolute bioavailability was determined to be approximately 160% in both groups. A bioavailability value >100% is not theoretically physiologically, but when extended duration drugs, such as CEF CFA, are administered they often result in a difference in clearance as compared to IV administration. This increases the exposure of the drug following extravascular administration, which increases the AUC resulting in this phenomenon. Therefore, CL via both routes of administration were introduced into the equation to account for potential difference. As a result, the apparent bioavailability was determined to be 98% and 110% for the DIS and CON groups. Plasma CEF C_{max} was lower and T_{max} was longer in this trial compared to the previous work (Chapters 2 and 3) due to flip-flop kinetics associated with CEF CFA when administered at the base of the ear per label compared to CEF hydrochloride.

Another potential change in clinical practice compared to the drug approval process is the co-administration of two or more drugs to alleviate the symptoms of clinical disease. In the drug approval process, drugs are administered singly (US FDA, 2006b). However, in clinical practice antimicrobials are often administered with other drugs, such as steroidal and non-steroidal anti-inflammatories, fluids, electrolytes, and diuretics. A commonly administered drug combination is CEF and flunixin meglumine (Kissell et al., 2015; Schuler et al., 2017). Ceftiofur (S. A. Brown et al., 1991) and flunixin meglumine (Anderson et al., 1990; Odensvik et al., 1995) are both >90% protein-bound. Flunixin and CEF are weak acids for which protein-binding is primarily associated with albumin (Riviere, 2009a). The objective of Chapter 3 was to compare plasma and ISF PK between CEF and flunixin administered individually versus co-administration of the two drugs. It was hypothesized that simultaneous administration would result in a drug interaction that would alter the plasma or ISF PK of one or both drugs. The results of this trial indicated that drug interactions between flunixin and CEF did not occur when the two drugs are administered simultaneously in healthy cattle. However, as albumin concentrations typically decrease to compensate for the body's need to increase production of acute phase proteins in disease (Ceciliani et al., 2012), it would be enlightening to repeat this work in diseased cattle to discover if there are interactions that occur as albumin concentrations decrease.

This work did not prove the hypothesis that disease would result in alterations in CEF PK necessitating implementation of an extended meat withdrawal time. However, the evidence uncovered suggests that it is theoretically possible. Therefore, tissue residue depletion studies should continue in animals that are more severely affected than those in the residue depletion trial (Chapter 4).

Internationally, there is concern that administration of veterinary drugs is resulting in dissemination of antimicrobial resistant (AMR) bacteria to humans. There is particular concern with the use of CEF, as it is a third generation cephalosporin closely related to the human antimicrobial, ceftriaxone. Antimicrobial resistance mechanisms that result in CEF resistance are highly correlated with resistance to ceftriaxone. This is particularly concerning since ceftriaxone is often used in human medicine for the treatment of invasive enteric salmonellosis in children (Whichard et al., 2005; Tragesser et al., 2006). Concerns regarding the future efficacy of 3rd generation cephalosporins has led the World Health Organization to classify them as the highest priority, critically important antimicrobials for the preservation of human health (WHO, 2017). Additionally, the US Food and Drug Administration has restricted the extra-label use of CEF in an attempt to limit the impacts on AMR development (US FDA, 2012b).

Resistance to β -lactams is most commonly associated with the production of β -lactamase enzymes. Extended spectrum β -lactamases are the primary cause of AMR in isolates of *Enterobacteriaceae* on a worldwide basis (Bonnet, 2004; Zhao and Hu, 2013). Prior to 2010, resistance determinants amongst *Enterobacteriaceae* for CEF in dairy cattle have primarily been related to the *bla*_{CMY-2} gene. However, recent data suggests the *bla*_{CTX-M} is now the most prevalent cause of extended spectrum β -lactamase derived resistance (Daniels et al., 2009; Davis et al., 2015). The final objective of this dissertation was to enumerate total and resistant *E. coli* from fecal samples CEF following administration of CEF CFA versus non treated control animals, compare phenotypic antimicrobial resistance and underlying genetic mechanisms from isolated bacteria, and determine if there was an impact of clinical disease on

the development of AMR. The hypothesis was that administration of CEF would result in altered AMR patterns in diseased animals compared to healthy animals.

Following treatment with CEF, there was a decrease in total *E. coli* cfu count and an increase in CEF resistant *E. coli*. There was a significant effect of CEF treatment on resistant bacteria between the negative control and diseased group and a tendency for a difference between the healthy cows receiving CEF and diseased cows receiving CEF. Like prior studies have shown, changes in total and resistant *E. coli* populations were transient and returned to approximately pre-treatment levels by 14 days following therapy.

One hundred twenty-nine *E. coli* isolates were found to be CEF resistant. All of these were also ampicillin and ceftriaxone resistant and 64.3% were tetracycline resistant. This is particularly troubling as CEF, ampicillin, and the tetracycline family drugs are amongst the most commonly used drugs in the dairy industry (Schuler et al., 2017).

In total, 55 of 129 CEF resistant fecal isolates had at least one β -lactamase resistance gene detected by PCR. Forty-eight harbored the *bla*_{CTX-M} gene while nine harbored the *bla*_{CMY} gene, with two of these isolates harboring both genes. All of the cows treated with CEF shed *E. coli* that were positive for the *bla*_{CTX-M} gene at least one time during the first 21 days of the 28-day trial. Interestingly, no *E. coli isolate* had the *bla*_{CTX-M} gene on day 28 following treatment. Isolates that were *bla*_{CTX-M} positive were resistant to 4.7 antimicrobials, while those with the *bla*_{CMY} gene were resistant to 5.5 antimicrobials. Bacterial isolates that were resistant to CEF and did not contain either of the *bla* genes were resistant to 4.65 antimicrobials. In contrast, from the 93 isolates that were CEF susceptible there was a mean resistance to 1.1 antimicrobials/isolate.

Going forward, the dairy industry must continue to improve management practices in order to minimize the need for antimicrobial use. Additionally, implementation of prudent treatments by veterinarians and the use of well-defined treatment protocols by lay personnel on farms is an essential goal to minimize drug use, especially since there are many conditions in which the use of antimicrobials is not indicated. Antimicrobials are essential for the treatment of many disease conditions in order to preserve animal welfare. However, if veterinarians do not take the lead on implementing prudent antimicrobial stewardship programs, the consuming public is going to demand dairy products that have been produced without the use of any antimicrobials.

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APPENDIX A: A STUDY TO EXAMINE THE RELATIONSHIP BETWEEN METRITIS
SEVERITY AND DEPLETION OF OXYTETRACYCLINE IN PLASMA AND MILK
AFTER INTRAUTERINE INFUSION

An abstract presented at the 2015 American Association of Bovine Practitioners Annual Conference and with full manuscript published in J. Dairy Sci. 2016, 99:8314–8322.

Abstract

Metritis is a frequent problem in post-partum dairy cows. Intrauterine therapy (IU) with the antimicrobial oxytetracycline (OTC) is often used, although efficacy data supporting this therapy has not been shown to be superior to systemic therapy. The objectives of this study were to (1) use high-pressure liquid chromatography coupled with mass spectrometry technology (LC-MS) to determine the plasma and milk concentrations of OTC following IU infusion in post-partum dairy cows with varying degrees of metritis severity, (2) determine the depletion time of OTC in an attempt to provide veterinarians withdrawal guidelines should they utilize this therapy, and (3) correlate metritis severity scores with OTC concentrations in plasma and milk. Our hypothesis was that cows with more severe metritis, as assessed by metritis scores, would have higher OTC concentrations in milk following IU therapy. Thirty-two cows were selected to participate in the study after farm personnel had determined the cows had metritis based on their evaluation of the character of vaginal discharge between 4 -14 days in milk, in accordance with the farm's treatment protocols. Metritis scores (1 - 4) were assigned as follows based on a published scheme where; (1) represented a yellow to orange thick discharge or translucent mucus, no fetid smell; (2)

represented a blood tinged vaginal mucus, slightly watery, little or no fetid smell; (3) represented a red to red/brown watery discharge; moderate fetid smell and (4) represented a red to red/brown watery discharge containing pieces of placenta and an intense fetid smell. Trial cows received a single treatment of 4 grams of OTC (approximately 6.7 mg/kg) via IU infusion. Blood samples were collected over 96 h and milk samples were collected prior to IU therapy and three times a day for 4 days post-infusion. Following treatment, OTC rapidly diffused to plasma and subsequently to milk. Maximum OTC concentration in plasma and milk occurred within the first 24 h following IU infusion and 25 of the 32 cows had detectable OTC concentrations in milk at four days after IU infusion. Cows with metritis scores consistent with clinical metritis (score=3 or 4) at the initiation of treatment were significantly, and positively, correlated with higher milk OTC concentration at the second milking (T9 h) ($R^2 = 0.43$), fourth (T25 h) ($R^2 = 0.42$) and fifth following treatment (T33 h) ($R^2 = 0.38$) milking following treatment compared to cows with normal vaginal discharge. There was also a positive correlation between an initial metritis score and milk maximum concentration ($R^2 = 0.36$) and also milk area under the concentration curve ($R^2 = 0.36$). Given that IU administration of OTC is considered to be an extra-label therapy, dairy producers should consult with their veterinarian to ensure that milk is being tested at or below the established tolerance for OTC. This will ensure that violative drug residues do not enter the human food supply.

APPENDIX B: COMPARISON OF MILK AND PLASMA PHARMACOKINETICS OF MELOXICAM IN POST-PARTUM VERSUS MID-LACTATION HOLSTEIN COWS

An abstract presented at the 2017 American Association of Bovine Practitioners Annual Conference and with full manuscript submitted for publication in *J. Vet. Pharmacol. Ther.*, 2017.

Abstract

Recently, researchers determined that treatment of post-partum dairy cows with meloxicam resulted in increased milk production throughout the entire lactation (Carpenter et al, 2016). However, according to the Animal Medicinal Drug Use Clarification Act (AMDUCA), this type of practice would not be allowed unless the practice was a therapeutic use (US FDA, 1996). The objective of this study reported here was determine if differences occurred in meloxicam pharmacokinetics between post-partum cows and mid-lactation cows. Three different groups were enrolled, each with 10 cows. The treatment group (TRT) was post-partum cows treated with meloxicam; 2) the negative control (NC) group was post-partum that received a placebo; and 3) the positive control (PC) group was cows in mid-lactation treated with meloxicam. Plasma and milk meloxicam concentrations between the TRT and PC group were compared. Significant differences in meloxicam concentration in plasma were determined at all time points from 8 h to 120 h post-treatment. In milk, there was a treatment ($P=0.003$), time ($P<0.001$), and treatment by time interaction ($P<0.001$). Significant differences in milk meloxicam concentration were determined at all time points from 8 h to 96 h post-treatment, except for the 16 h time point. The time needed for

meloxicam to no longer be detected in milk of the TRT group was longer compared to the PC group, indicating that a longer milk withdrawal is needed. These data suggest higher bioavailability as the underlying mechanism. Further research is needed to determine the mechanisms underlying differences this outcome.

APPENDIX C: SURVEY OF TREATMENT PRACTICES ON MIDWEST DAIRY FARMS

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Abstract

The objective of this study was to assess current antimicrobial use practices and veterinarian involvement with these practices on upper Midwest dairy farms. Eighty-five dairy farms ranging in size from 105 to 5,400 lactating cows located in six states (SD, NE, IA, MN, WI, and IL) were visited by two veterinary students during the summer of 2015. Interns observed farm treatment practices, reviewed individual herd treatment protocols and records, and conducted a standardized survey with farm management assessing mastitis, metritis, lameness, pneumonia, heifer pneumonia, and heifer diarrhea. Results indicate the presence of written treatment protocols varied by disease type. Metritis was the most common disease for which a protocol was found on-farm (49%), followed by mastitis (46%), lameness (39%), adult cow pneumonia (34%), heifer pneumonia (21%), and heifer diarrhea (19%). Ceftiofur was the most common primary antimicrobial selected for the treatment of mastitis (61%), metritis (82%), lameness (54%), and pneumonia (72%). Thirty-nine percent of farms selected enrofloxacin as their primary antimicrobial for the treatment of calf diarrhea. This use of enrofloxacin was also the most common unapproved treatment observed in the study. Results of this study demonstrate an opportunity for veterinarians to educate producers about judicious antimicrobial use on dairy farms.